

Clinical significance of the expression of DNA methyltransferase proteins in gastric cancer

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Abstract. DNA methyltransferase (DNMT) 1, DNMT3A and DNMT3B, which affect promoter CpG methylation status, play a significant role in cancer development. Little is known regarding the clinical significance of DNMT expression in gastric cancers. Expression of DNMT1, DNMT3A and DNMT3B in paraffin sections from 54 gastric cancer patients were examined using immunohistochemistry, and their associations with the corresponding clinicopathological parameters were analyzed using the Chi-square test. Overexpression of DNMT1, DNMT3A and DNMT3B in gastric cancer tissues was observed in 35 (64.8%), 38 (70.4%) and 28 (51.9%) of 54 cases, respectively. DNMT1 was localized in the cytoplasm and nuclei of the cancer cells, whereas DNMT3A and DNMT3B were detected only in the cytoplasm. DNMT1 expression was more frequently found in tumors localizing at the cardia or body of the stomach ($P=0.048$). DNMT3A was associated with TNM stage ($P=0.001$) and lymph node metastasis ($P=0.002$). No significant correlation was found between DNMT3B expression and clinicopathological data ($P>0.05$). The co-expression of DNMT1 and DNMT3A, and of DNMT3A and DNMT3B was more frequently found in tumors localizing at the cardia or body of the stomach ($P=0.005$ and $P=0.009$ respectively). Moreover, co-expression of DNMT1 and DNMT3A was significantly associated with lymph node metastasis ($P=0.035$). DNMTs are overexpressed in gastric cancer, and may play a significant role in the development of aberrant promoter methylation during tumorigenesis.

Introduction

Carcinogenesis of gastric cancer is a multistep process triggered mainly by *Helicobacter pylori* and characterized by the accumulation of molecular alterations (1). Genetic and epigenetic alterations are two major events implicated in cancer-related molecular alterations. One of the epigenetic alterations during carcinogenesis is DNA methylation, which is characterized as the addition of a methyl group from S-adenosyl-L-methionine (SAM) to the carbon 5 position of the cytosine ring within the CpG dinucleotide (2). DNA methylation has been identified as a significant and alternative mechanism in tumorigenesis (3). It is required for the normal development of cells, whereas the aberrant methylation of CpG islands confers a selective growth advantage, resulting in tumor growth (1). Previous studies on gastric cancer identified a CpG island methylator phenotype, which involves the targeting of multiple tumor-suppressor genes (TSGs) such as RASSF2 (4), NMDAR2B (5), PRDM5 (6), BNIP3 (7) and CDH4 (8) by promoter hypermethylation (9-11). It has been confirmed that CpG island methylation of TSGs is associated with the development of esophageal carcinoma (12,13). Epigenetic silencing of these genes may also facilitate gastric tumorigenesis. We therefore suggest that the aberrant expression of DNMTs leads to tumorigenesis. CpG methylation is mediated by at least three active DNA methyltransferases (DNMTs). DNMT1 has been postulated to perform maintenance of methylation. DNMT3A and DNMT3B were previously identified as enzymes that facilitate *de novo* methylation (14). The three enzymes cooperate in establishing and maintaining DNA methylation patterns (15). Therefore, DNMTs, which involve DNA methylation, play a significant role in the regulation of gene expression. However, the expression of DNMTs and their involvement in gastric cancer remain unclear. To gain more insight into the contribution of the expression of DNMTs in gastric cancer, we examined the expression of DNMT1, DNMT3A and DNMT3B in gastric cancer patients and compared their expression status with the corresponding clinicopathological data. We aimed to provide a beneficial approach to the epigenetic theory of gastric cancer development, thus offering a basis of early diagnosis and a novel treatment for gastric cancer.

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Table I. Basic clinicopathological information of the 54 patients and correlations between the immunoreactivity of DNMTs and clinical parameters in gastric cancer.

Clinicopathological parameters	No. of cases	DNMT1 protein expression			DNMT3A protein expression			DNMT3B protein expression		
		T>N	T≤N	P-value	T>N	T≤N	P-value	T>N	T≤N	P-value
Age (years)										
≤60	28	20	8	0.291	18	10	0.31	14	14	0.777
>60	26	15	11		20	6		12	14	
Gender										
Male	41	28	13	0.537	29	12	1	21	20	0.422
Female	13	7	6		9	4		5	8	
Tumor location										
Cardia and body of stomach	24	19	5	0.048	20	4	0.062	14	10	0.18
Gastric antrum	30	16	14		18	12		12	18	
TNM stage										
I/II	25	15	10	0.492	12	13	0.001	12	13	0.984
III/IV	29	20	9		26	3		14	15	
Tumor differentiation										
Poorly differentiated	36	21	15	0.158	25	11	0.833	15	21	0.178
Well- or moderately differentiated	18	14	4		13	5		11	7	
Lauren typing										
Intestinal type	29	21	8	0.208	19	10	0.4	16	13	0.266
Diffuse type	25	14	11		19	6		10	15	
Lymph node metastasis										
Yes	34	24	10	0.247	29	5	0.002	16	18	0.835
No	20	11	9		9	11		10	10	

T, cancer tissue; N, normal tissue.

Materials and methods

Patients and tissue samples. A total of 54 paraffin sections of gastric cancer were obtained from the Department of Pathology of the Affiliated Nanjing First Hospital, Nanjing Medical University, China. The patients were pathologically confirmed as presenting primary gastric cancer between September 2006 and November 2008, and none of them received radiotherapy or chemotherapy prior to surgery. The basic clinicopathological characteristics for the 54 patients are shown in Table I. The tumor and adjacent non-tumor tissues were processed immediately after the operation and fixed in buffered paraformaldehyde. The adjacent non-tumor tissue was dissected at least 5 cm from the tumor edge and considered as comparably normal for the control. The gastric cancer patients provided written informed consent on the use of clinical specimens for medical research.

Immunohistochemistry. Immunohistochemistry was performed according to the streptavidin-biotin complex (SABC) method (16,17). The sections were baked at 60°C for 15 min before being dewaxed in xylene and rehydrated

in graded alcohol. Slides filled with sodium citrate buffer (pH 6.0) were preheated for 8 min and boiled for 15 min in a microwave oven for antigen retrieval. Endogenous peroxidase activity was blocked using 3% H₂O₂ in phosphate-buffered saline. Equinum serum was used to block non-specific protein binding for 30 min. The primary antibody was added and sections were incubated overnight at 4°C for DNMT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-10219; 1:200 dilution), DNMT3A (Imgenex, San Diego, CA, USA; IMG-268A; 1:75 dilution) and DNMT3B (Santa Cruz SC-10235, 1:150 dilution). This process was followed by the addition of the secondary antibody (KPL, Lot no. WJ137, 1:200 dilution) and ABC reagent and DAB as a chromogen. After washing, slides were counterstained with hematoxylin, dehydrated in graded series of ethanol and mounted with Pertex (Histolab, Denmark). The SABC kit was purchased from Fuzhou Maixin Biological Technology (Fuzhou, Fujian, China), and the DAB kit was purchased from Beijing Zhongshan Company. Nuclear and cytoplasmic immunopositivity was evaluated using the scoring system according to the staining intensity (0, negative; 1, mild; 2, moderate; and 3, strong) and the proportion of positive cells (0, negative; 1,

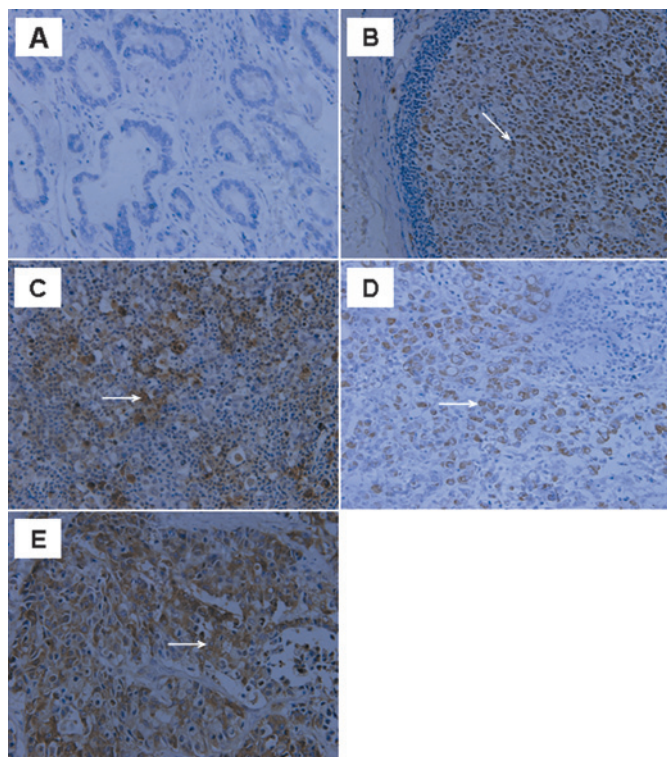


Figure 1. DNMTs in gastric cancer tissues; SABC method; magnification, x400. Color of the staining is brown. Compared with (A) the negative group, DNMT1 was well distributed in (B) the nuclei (↖), or (C) cytoplasm (→) of the cancer cells. (D and E) DNMT3A (→) and DNMT3B (→), respectively, were both well distributed in the cytoplasm.

≤10%; 2, 10%-1/3; 3, 1/3 -2/3; and 4, >2/3). The two scores were then combined for each slide, and the immunoreactivity of the DNMT protein was graded as follows: 0, negative (-); 1-2, weakly positive (+); 3-5, moderately positive (++); or 6-7, strongly positive (+++). If the score obtained from the tumor tissue was greater than that from the non-tumor tissue, it was defined as overexpression of DNMT. The rating results of immunohistochemistry for all tissue slices were viewed according to the above criteria by pathologists.

Statistical analysis. Statistical analyses were carried out using Stata (version 8.2; StataCorp LP, College Station, TX). Correlations between the immunoreactivity of the DNMTs and the clinicopathological parameters (age, gender, location of the tumor, TNM stage, tumor differentiation, Lauren typing, lymph node metastasis and nerve and blood vessel invasion) in gastric cancer tissues were analyzed using the Chi-square test. The results were considered statistically significant at $P < 0.05$.

Results

Protein expression of DNMTs in gastric cancer tissues. Overexpression of DNMT1, DNMT3A and DNMT3B in gastric cancer tissues was observed in 35 (64.8%), 38 (70.4%) and 28 (51.9%) of 54 cases, respectively, indicating that an aberrant DNA methylation pattern was present in the gastric cancer tissues (Table I). The immunoreactivity of DNMT1 was found in the cytoplasm or nuclei of the cancer cells, whereas

Table II. Correlation between the co-expression of DNMT1 and DNMT3A and clinical parameters in GC tissues.

Clinicopathological parameters	DNMT1 and DNMT3A co-expression		P-value ^a
	T>N	T≤N	
Tumor location			
Cardia and body of stomach	18	6	
Gastric antrum	11	19	0.005
Lymph node metastasis			
No	7	13	
Yes	22	12	0.035

^aChi-square test; T, cancer tissue; N, normal tissue.

Table III. Correlation between the co-expression of DNMT3A and DNMT3B and clinical parameters in GC tissues.

Clinicopathological parameters	DNMT3A and DNMT3B co-expression		P-value ^a
	T>N	T≤N	
Tumor location			
Cardia and body of stomach	12	12	
Gastric antrum	5	25	0.009
Lymph node metastasis			
No	4	16	
Yes	13	21	0.164

^aChi-square test; T, cancer tissue; N, normal tissue.

that of DNMT3A and DNMT3B were observed only in the cytoplasm (Fig. 1).

Correlation between DNMTs and clinical parameters in gastric cancer tissues. DNMT1 overexpression was significantly associated with tumor location ($P=0.048$). However, marked associations were present between DNMT3A immunoreactivity and TNM stage ($P=0.001$) and lymph node metastasis ($P=0.002$). No significant correlation was found between DNMT3B expression and the clinicopathological data ($P>0.05$) (Table I).

Correlation between the co-expression of DNMTs and clinical parameters in gastric cancer tissues. Co-expression of DNMT1 and DNMT3A was more likely to be found in the cardia or body of the stomach than in the gastric antrum ($P=0.005$), and was associated with lymph node metastasis ($P=0.035$, Table II). Thus, the co-expression of DNMT3A and DNMT3B correlated significantly with tumor location ($P=0.009$, Table III).

Discussion

Gastric cancer is one of the leading causes of mortality in both men and women in China. The progression of gastric cancer involves multiple steps, including overexpression of oncogenes and inactivation of TSGs. In addition to genetic alterations, aberrant gene expression is also attributed to epigenetic changes including methylation and hypomethylation, which do not necessarily change DNA sequences but have an effect on gene expression levels. Currently, the mechanisms by which DNA methylation is responsible for increased cancer risk have been the subject of attention. DNA methyltransferases, as the indispensable factor in the process of DNA methylation, have been widely discussed.

Previous studies have revealed that methylation patterns become abnormal in malignant cells and that improper methylation partially induces tumorigenesis (18). DNMT3A and DNMT3B are *de novo* DNA methyltransferases that modify unmethylated DNA. By contrast, DNMT1 exhibits high preference for hemimethylated DNA; however, numerous experiments have found that DNMT1 was activated by the pre-existing methyl groups, which is a result of *de novo* methylation by DNMT3 (15,19). We therefore suggest that DNMT1 is also involved in the *de novo* methylation of DNA. Mounting evidence has shown that the three DNA methyltransferases collaborated with one another and played a key role in tumorigenesis (20-24) regarding neoplastic proliferation (24).

Overexpression of DNMTs is commonly found in various tumor types. For example, overexpression of the DNMT3B protein was observed in human breast cancer cell lines (25) and breast tumors (26), contributing to the elevated DNMT3B activity. Nagai *et al* (27) suggest that the overexpression of DNMT1 and DNMT3B is involved in hepatocellular carcinogenesis. Similar overexpression patterns of DNMT1, DNMT3A and DNMT3B in gastric cancer have been reported by Ding *et al* (28). In their patient cohort, the expression of DNMT1, DNMT3A and DNMT3B in tumor tissue was 81.6, 81.6 and 68.4%, respectively. This expression was significantly higher compared to that for para-cancerous tissues (39.5, 50 and 44.7%). The proportions obtained for the expression of DNMTs (64.8, 70.4 and 51.9%, respectively, for DNMT1, DNMT3A and DNMT3B in gastric cancer tissues) were lower compared to those observed in the study by Ding *et al*, but also confirmed that the aberrant overexpression of DNMTs is present in gastric cancer tissues. However, Robertson *et al* (18) revealed that only DNMT3B was significantly overexpressed, with lower frequencies in tumors whereas DNMT1 and DNMT3A were modestly overexpressed. Further studies are required to include larger numbers of cases in order to investigate the expression of the three DNA methyltransferases in gastric cancer tissues.

On the other hand, the location of DNMTs in cells indicates the manner of methylation of the respective DNMTs. In B spermatogonia and resting fibroblasts, Galetzka *et al* observed DNMT3A foci in the nucleus (29). Our experiment revealed that the immunoreactivity of DNMT1 was well distributed in the cytoplasm or nuclei of the cancer cells, whereas DNMT3A and DNMT3B were well distributed only in the cytoplasm. Therefore, we believe that the proteins belonging to the DNMT3 family in the cytoplasm are involved in *de novo* methylation, which is consistent with a known

transcription process in the cell nucleus, whereas the translation process occurs in the cytoplasm.

Due to the overexpression of DNMTs in gastric cancer, we hypothesized that there were associations between the expression of DNMTs and clinical parameters in gastric cancer tissues. Analysis of the correlation between DNMTs and clinical parameters of gastric cancer patients provides an understanding of the occurrence and development of the disease. On the other hand, DNMT1 and DNMT3B cooperatively maintain DNA methylation and gene silencing in human cancer cells (24). Disruption of the human DNMT3B only slightly reduces global DNA methylation; however, demethylation was markedly potentiated when DNMT1 and DNMT3B were simultaneously deleted (30). Due to the close relationship between DNMTs, we investigated and produced an assay of single and co-expression of DNMTs and patient clinical parameters.

The relationship between the expression of DNMTs and clinical parameters for certain types of cancer, including gastric cancer, has already been reported. In their study, Ding *et al* (28) showed that the DNMT1 protein expression exhibited no correlation with age, lymph node metastasis or tumor differentiation but may have had a correlation with gender, whereas the DNMT3 family was not associated with these factors. In contrast with the study by Ding *et al*, we obtained different results in the present report in that DNMT1 was overexpressed in the cardia or body of the stomach, as well as the co-expression of DNMT1 and DNMT3A, or DNMT3A and DNMT3B. Moreover, there were marked associations between DNMT3A immunoreactivity, III/IV TNM stage and lymph node metastasis. Combined with an analysis of clinicopathological characteristics, these correlations indicate that DNMT3A is involved in the evolution and metastasis of gastric cancer. A correlation between the co-expression of DNMT1 and DNMT3A and lymph node metastasis may therefore be identified. Similarly, Choi *et al* (31) proposed that the dysregulation of DNMT3A may be involved in the progression of hepatocellular carcinoma. It is therefore likely that DNMT3A plays a critical role in tumor progression and the extensional mechanism requires further investigation. We also revealed no link between the clinical parameters and immunoreactivity of DNMT3B.

In conclusion, our study indicated that DNMTs were overexpressed in gastric cancer tissues. Although overexpression of DNMTs is not equal to the aberrant DNA methylation pattern in gastric cancer, our findings, such as the distribution of DNMTs and the correlations between DNMTs and clinical significance, may prove beneficial. The test for detecting the level of DNMTs may facilitate the prediction of patient prognosis, and play a crucial role in the treatment of gastric cancer. Furthermore, results may be improved by increasing the sample size, or by performing a meta-analysis to gain the benefits of evidence-based science. On the other hand, further studies are required to examine the relationship between the overexpression of DNMTs and aberrant DNA methylation, and the mechanism involved.

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