Clinical applications of DNA methylation in gastrointestinal cancer

Maat, M.F.G. de

CHAPTER 5

Epigenetic Silencing of Cyclooxygenase-2 Affects Clinical Outcome in Gastric Cancer

Michiel F.G. de Maat[a,c], Cornelis J.H. van de Velde[c], Naoyuki Umetani[a], Pieter de Heer[c], Hein Putter[d], Anneke Q. van Hoesel[a,c], Gerrit A. Meijer[e], Nicole C. van Grieken[e], Peter J.K. Kuppen[c], Anton J. Bilchik[b], Rob A. E. M. Tollenaar[c], and Dave S. B. Hoon[a]

[a]Department of Molecular Oncology and [b]Division of Gastrointestinal Surgery, John Wayne Cancer Institute, Santa Monica, CA, USA
Departments of [c]Surgery and [d]Medical Statistics and Bioinformatics, Leiden University Medical Center, The Netherlands
[e]Department of Pathology, VU University Medical Center, Amsterdam, the Netherlands

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Abstract

Purpose: Overexpression of cyclooxygenase-2 *(COX-2)* in gastric cancer has been shown to enhance tumor progression. We investigated whether silencing by promoter region hypermethylation of the *COX-2* gene contributes to disease outcome in gastric cancer.
Materials and Methods: *COX-2* methylation status was initially assessed by capillary array electrophoresis methylation-specific PCR (CAE-MSP) and COX-2 protein expression by immunohistochemistry (IHC) in 40 primary gastric cancer tissues in a pilot study. Prognostic endpoints of correlative studies of *COX-2* methylation status were time to recurrence, overall survival, and standard clinicopathologic features. CAE-MSP analysis was then validated in a second independent gastric cancer population (n=137).
Results: *COX-2* methylation was detected in 23% and 28% of the pilot and validation patient groups, respectively. *COX-2* expression (IHC) in gastric tumors inversely correlated with *COX-2* gene methylation status in the pilot study (P=0.02). *COX-2* methylation in tumors was significantly associated with lower T-, N-, and TNM-stage in the validation patient group (P=0.02, P=0.006 and P=0.008, respectively). Patients with *COX-2* methylated tumors had significantly longer time to recurrence (TTR) and improved overall survival (OS) in a multivariate analysis in the smaller (HR=0.08; 95%CI, 0.01-0.65 and HR=0.37; 95%CI, 0.14-1.00, respectively) and larger patient groups (HR=0.49; 95%CI, 0.24-0.99 and HR=0.62; 95%CI, 0.38-0.99, respectively).
Conclusion: Hypermethylation of *COX-2* gene promoter was identified as an independent prognostic factor in gastric cancer patients. The results suggest promoter hypermethylation to be an important regulatory mechanism of *COX-2* expression in gastric cancer and show a beneficiary effect of tumor-related methylation on disease outcome in gastric cancer patients.

Introduction

Despite its decreased incidence, gastric cancer remains the second most common cause of cancer death and the third most common cancer worldwide[1]. Recently, gastric cancer therapy has received more attention, since neoadjuvant modalities have shown to improve outcome for resectable tumors[2-4]. Molecular surrogate marker(s) of disease outcome could be of benefit in the management of gastric cancer patient treatment.

About 60% of human genes are associated with clusters of CpG dinucleotides, referred to as CpG islands[5]. Clustered methylation of CpG islands at a gene promoter or transcription start site is associated with gene silencing. This epigenetic event has been observed in many genes of different cancers[6]. Hypermethylation of tumor-related regulatory genes may play a significant role in tumor transformation and progression, impacting the clinical course of disease. Recent studies focus on hypermethylation of specific tumor-related genes with suppressing capacities on growth and proliferation of gastric cancer[7-11]. Genes regulated by methylation status can have tumor suppressor functions, as well as tumor-inducing capacities, significantly altered. Therefore, gene inactivation by hypermethylation may have dual effects on tumorigenesis and tumor progression. Epigenetic inactivation of genes related with tumor progression has not been well studied in gastric cancer as related to disease outcome.

COX-2 (cyclooxygenase-2/*PTGS2*, prostaglandin-endoperoxide synthetase-2) expression is upregulated in gastrointestinal cancers[12-15]. In gastric cancer, *COX-2* expression is involved in several important tumor progression-related mechanisms, such as angiogenesis[16], inhibition of apoptosis[17], and invasiveness[18]. Song et al. demonstrated regulation of *COX-2* mRNA and protein expression by hypermethylation of the *COX-2* promoter region in gastric cancer lines[19]. Most gastric cancers overexpress *COX-2*, and, recently, *COX-2* expression assessed by immunohistochemistry (IHC) was identified to impact disease survival[20]. Because of the reported epigenetic regulation and predictive value of *COX-2* expression, we hypothesized a role for *COX-2* promoter hypermethylation status in the clinical outcome of patients with gastric cancer. To study this, we first assessed *COX-2* promoter methylation status by quantitative methylation-specific PCR (MSP), as well as its relation to *COX-2* protein expression in paraffin-embedded archival tumor (PEAT) specimens of gastric cancer patients with known disease outcome in a pilot study. All patients were enrolled in a randomized, multi-center trial for primary gastric cancer comparing preoperative chemotherapy versus surgery alone[21,22]. The clinical impact of *COX-2* methylation in the cancer trial patients was studied by correlating disease outcome to tumor *COX-2* methylation status. The MSP findings were then confirmed in a larger, independent validation patient group, selected from another multi-center randomized trial comparing primary tumor resection with limited versus extended nodal dissection[23,24].

Materials and Methods

Tumor specimens

The pilot study group contained patients (n=59) accrued in the FAMTX (5-fluorouracil,

doxorubicin, and methotrexate) trial conducted by the Dutch Gastric Cancer Group (DGCC)[21,22] evaluating preoperative chemotherapy with FAMTX for gastric cancer. As a validation set, patients were used from the D1D2 trial by the DGCC[23,24]. The trial evaluated (sub)total gastrectomy for gastric cancer with D1 to D2 lymph node dissection of which the latter, included partial removal of spleen and pancreas. All tumors were classified and staged according to the revised guidelines set by the International Union Against Cancer (UICC). PEAT specimens of the primary tumor were collected from patients from both trials. PEAT specimens from gastric tissue biopsies for benign conditions as controls were also collected from 18 patients without a history of malignancy. The protocol for this study was approved by the Human Subjects Institutional Review Boards of both participating institutions (Saint John's Health Center / JWCI; Leiden University Medical Center).

Tissue and DNA preparation

Serial sections from each PEAT specimen were cut. One section (4 µm) was stained by hematoxylin and eosin and a tumor representative tissue was marked by an expert surgical pathologist for gastric cancer (G.A.M.). The next section (7 µm) was deparaffinized, and lightly stained with hematoxylin. Tumor tissue was precisely isolated by manual microdissection under an inverted microscope using the marked HE section for target tissue identification. Isolated tissue was digested by 50 µl of proteinase K (Qiagen Inc, Valencia, CA) containing lysis buffer at 50°C for 16hrs. Subsequently, DNA was purified with phenol-chloroform-isoamyl alcohol (Fisher Chemicals, Fairlawn, NJ) and precipitated by ethanol. Subsequent tissue sections (4 µm) were prepared on aminopropylethoxysilane (APES) coated slides for IHC.

Analysis of CpG island methylation status

Sodium bisulfite modification (SBM) was performed on 20 µl of sample PEAT DNA plus 1 µg of salmon sperm DNA as a carrier. Sample concentrations of double-stranded DNA were quantified by the PicoGreen assay (Molecular Probes, Eugene, OR) prior to bisulfite treatment and were between 10 and 100 ng/µl. We allowed for 90% sample loss during desalting and further clean-up steps. DNA isolation was repeated if concentrations were lower than 10 ng/µl. If insufficient DNA could be detected the sample was not further evaluated. SBM was carried out as previously described[25]; sulphonation incubation time was 3hrs at 60°C. Capillary array electrophoresis analysis was used after methylation-specific PCR for analysis of CpG island methylation status as previously described. Methylation-specific and unmethylated-specific primer sets were designed around the *COX-2* transcription start site (-14bp/+110bp). The primers were dye-labeled for detection using capillary array electrophoresis (CAE). Forward and reverse sequences for the methylation-specific primer set were: 5'-TTTCGGTTAGCGATTAATTGTTATAC-3' and 5'-CGAAAATAAACTTTAC-TATCTAAAAACGTC-3', respectively. Forward and reverse sequences for the non-methylated-specific primer set were: 5'-TTTGGTTAGTGATTAATTGTTATATGA-3' and 5'-CAAAA-ATAAACTTTACTATCTAAAAACATC-3', respectively. Two positive (*sssI methyltransferase* treated donor lymphocyte DNA and the RL-0380 cell line DNA), and two negative (*phi-29 DNA polypmerase* amplified donor lymphocyte DNA[26] and the FN-0028 cell line DNA) controls were included in each assay. Relative amounts of PCR products were quantified

by CAE (CEQ 8000XL, Beckman Coulter, CA) using CEQ 8000XL software version 6.0 (Beckman Coulter), as described previously[27]. A methylation index (MI) was calculated; MI = [(methylated peak intensity) / (methylated peak intensity + unmethylated peak intensity)][28].

Immunohistochemistry

Tissue sections were deparaffinized and endogenous peroxidase was blocked by hydrogen peroxidase-methanol for 20min. Antigen retrieval was performed by boiling the sections in 10mM citrate buffer for 10min. Sections were incubated overnight at room temperature with a monoclonal antibody against human *COX-2* (Cayman Chemical, MI) at a dilution of 1:200 (2.5 µg/mL) in phosphate-buffered saline (pH 7.4) with 1% BSA (PBS/BSA). Sections were then incubated for 30min with biotin (1:400; DAKO, Glustrup, Denmark), washed, and incubated for 30min with Streptavidin-Biotin-Complex (SABC) (1:100; DAKO, Denmark). The sections were washed in PBS for 15min, rinsed in Tris/HCl-buffer (pH 7.6) for 5 min, and developed in 3.3 diaminobenzidine tetrahydrochloride (DAB) with hydrogen-peroxide for 10min. The sections were counterstained with hematoxylin and mounted. *COX-2* IHC staining intensity of tumor cell cytoplasm was scored independently in a blinded manner by two expert gastric cancer pathologists (GAM and NCvG) using the following scoring criteria: absent staining; weak diffuse cytoplasmic staining (may contain stronger intensity in <10% of the cancer cells); moderate granular cytoplasmic staining in 10%-90% of the cancer cells; and strong granular staining in more than 90% of the tumor cells according to the method of Buskens et al[29]. In case of disagreement, a third independent staining assessment (by PdH) was used to designate tumor staining-intensity.

MSP assay validation

Methylation status of the *COX-2* promoter region was confirmed in gastric cancer lines, KATO-III (ATCC, Manassas, VA) and FN-0028 (JWCI), by direct bisulfite sequencing, as described previously[28]. Forward and reverse sequencing primers were 5'-TAAGGGGAGAGGAGGGAAAA-3' and 5'-CACCTATATAACTAAACYCCAAAACC-3', respectively, with Y=A or G. Both cell lines were treated with 5-azacytidine (5-aza) and trichostatin-A (TSA) for verification of epigenetic regulation of *COX-2* mRNA expression, as described previously[28,30]. *COX-2/GAPDH* mRNA expression ratio was assessed by using quantitative RealTime PCR[31]. Sequences for forward and reverse primers and fluorescent labeled probe for *COX-2* mRNA were 5'-CATTTGAAGAACTTACAGG-3', 5'-CCAAAGATGGCATCTG-3', and 5'-FAM-CTCCACAGCATCGATGTCACCATA-BHQ-3', respectively.

Study design and statistical analyses

This was a retrospective study and all assays were performed in a blinded manner to the trial clinical outcome parameters. We first established tumor-specific MI by assessment of non-neoplastic gastric tissue controls. A cut-off MI to allocate tumors to the methylated or unmethylated category was set at the 95th percentile of the measured MI values in normal controls. This cut-off was uniformly and consistently used to study the clinical value of *COX-2* methylation status initially in the pilot study and then in the validation D1D2 trial specimens. D1D2 trial patients that received resection with curative intent were selected,

satisfying the following criteria: complete surgical resection (R0) and no postoperative mortality. Required sample size of the validation patient group was calculated based on recurrence-percentages in patients with *COX-2* methylated and unmethylated tumors after 10 years of follow up of in the pilot study. Correlation between methylation status of the *COX-2* gene and clinicopathological features was analyzed by Fisher's exact test or Pearson's χ^2 test. Student's t-test evaluated differences in age between groups. The Mann-Whitney U-test was used for ordinal variables. Survival length was determined from the day of primary tumor surgery to the date of death or last clinical follow-up. The Kaplan-Meier method was used for survival analysis grouping with *COX-2* methylation status. Differences between curves were analyzed using the log-rank test. Cox's proportional hazard regression model was used in a backward stepwise method for variable selection in multivariate analyses. T-stage, N-stage, TNM-stage, trial randomization, Lauren classification, and complete resection were included in the model. Kruskal-Wallis test was used to assess the relation between *COX-2* MI and the different staining-intensity categories. The statistical package SPSS version 12.0.1 (SPSS Inc, IL) was used; a value of $P<0.05$ (two-tailed) was considered significant.

Results

MSP assay validation

Regulation of *COX-2* expression by promoter region methylation has previously been shown in gastric cancer lines[19]. We first verified whether the CAE-MSP assay on *COX-2* methylation status associated with regulation of *COX-2* mRNA expression. Two representative gastric cancer lines assessed by the CAE-MSP assay as completely methylated (KATO-III, MI=1.0) and completely unmethylated (FN-0028, MI=0). The individual CpG-dinucleotide methylation status of the target region of the promoter transcription site was confirmed by bisulfite sequencing in RL-0380 and FN-0028 to be, respectively, completely methylated and unmethylated. This established these cell lines as suitable positive and negative controls for the CAE-MSP assay. RL-0380 and FN-0028 cell lines were than treated by 5-aza and TSA to evaluate *COX-2* mRNA re-expression. The combination of 5-aza and TSA was used because epigenetic silencing can involve both methylation of CpG islands and deacetylation. In KATO-III, the demethylating effect was confirmed by CAE-MSP, and expression of *COX-2* mRNA was induced (*COX-2/GAPDH* ratio was 0 versus 4.46E-02, respectively, before and after treatment). In FN-0028, *COX-2* mRNA was present before treatment and did not significantly change after treatment (*COX-2/GAPDH* ratio was 1.69E-04 versus 3.45E-04, respectively). Results confirmed that promoter region methylation affects *COX-2* expression, and validated the CAE-MSP assay for detecting methylation at the *COX-2* promoter region transcription site.

Primary tumor COX-2 methylation status

Increased *COX-2* methylation has been shown to be a tumor-related event in gastric cancer[32,33]. Using the *COX-2* CAE-MSP assay, we verified tumor-related *COX-2* levels. Methylation status was assessed in FAMTX patients' primary tumors, as well as 18 non-

cancerous gastric biopsies in patients with benign conditions as controls. Forty-four of the 59 patients enrolled in the trial finally underwent resection. Three of the 44 primaries could not be evaluated because of insufficient DNA, and in one patient the paraffin-embedded block had an insufficient amount of cells, leaving 40 patients available for analysis. Relatively low levels of *COX-2* methylation were detected in control samples compared to tumors (**figure 1**). The 95th percentile of the MI values in non-neoplastic gastric tissues was calculated (MI=0.24) and used as a cut-off to establish tumor-related methylation. *COX-2* methylation was detected by the CAE-MSP assay in 9 of 40 (23%) tumors using the predetermined cut-off level standard.

Primary tumor COX-2 methylation status and COX-2 IHC

The regulatory effect of *COX-2* promoter methylation in tumors was assessed by correlating *COX-2* gene MI values to intratumoral COX-2 protein expression in FAMTX trial pri-

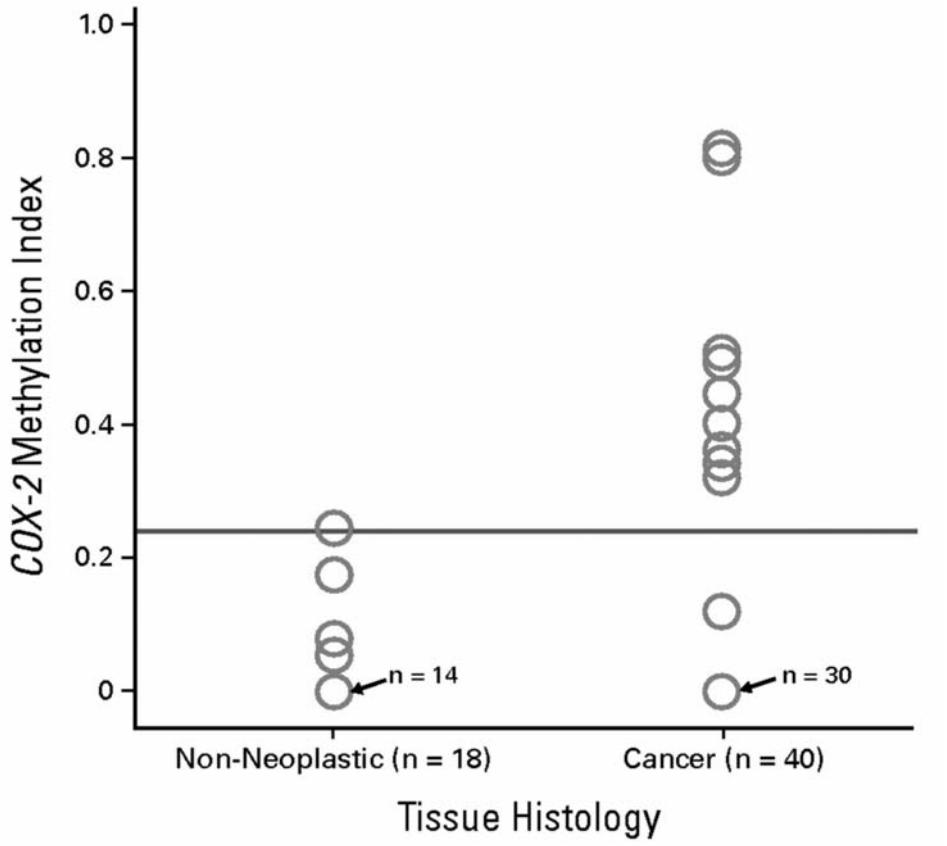

Figure 1. *Scatter plot indicating distribution of measured methylation index (MI) values in normal gastric epithelium and primary gastric tumor. Horizontal bar indicates the cutoff level for increased tumor-related methylation (MI, 0.24).*

maries. PEAT specimens were assessed by IHC for *COX-2* expression of tumor cells. Of the 39 evaluated cases, 3 (8%), 9 (23%), and 27 (69%) patients showed weak diffuse, moderate, and strong COX-2 expression, respectively. **figure 2** shows representative results of *COX-2* expression with the corresponding CAE-MSP signal intensity peaks. The relation of *COX-2* gene methylation status to respective protein expression in tumor cells is shown in **figure 3**. Mean MI values were 0.46, 0.32, and 0.13 for tumors with weak diffuse, moderate granular, and strong granular staining, respectively. The gradual decrease of methylation levels along *COX-2* IHC categories increasing in staining intensity was significant (P=0.02 by Kruskal-Wallis test), and suggests a direct regulatory effect of *COX-2* methylation on COX-2 protein expression in gastric tumors.

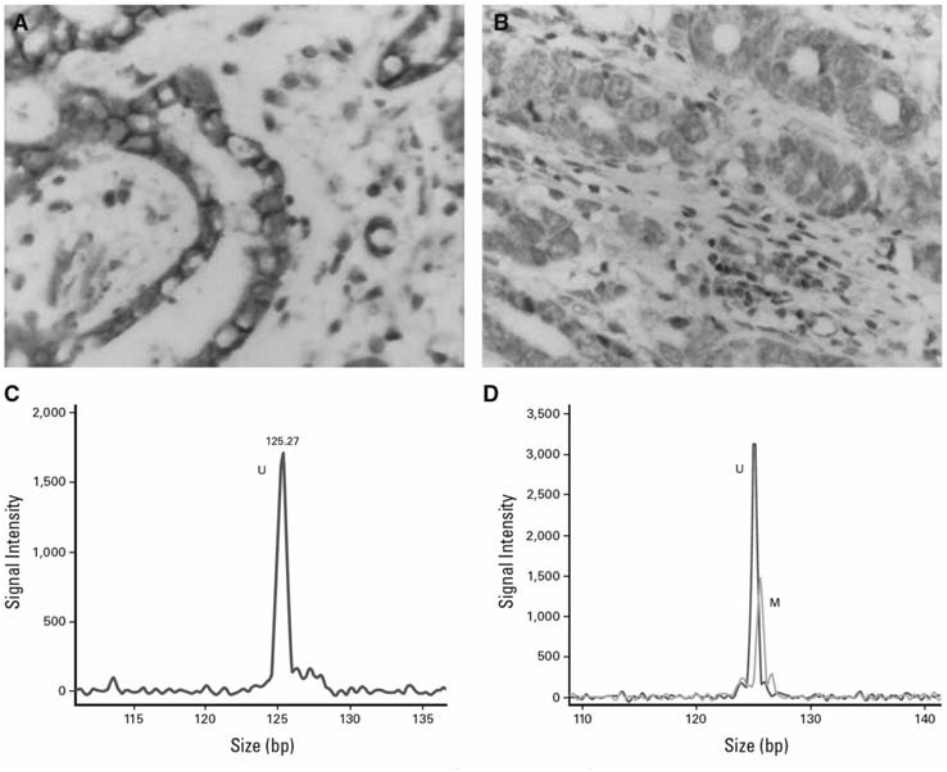

Figure 2. *Representative cyclooxygenase-2 (COX-2) immunohistochemistry (IHC) results of primary gastric tumors with respective capillary array electrophoresis methylation–specific polymerase chain reaction (CAE-MSP) outcomes. x-axis of the CAE-MSP represents the fluorescent intensity (M, methylated product; U, nonmethylated product) indicating polymerase chain reaction amplicon. y-axis represents the product base pairs. (A, C) Nonmethylated primary gastric tumor. Strong cytoplasmic COX-2 protein staining in tumor cells. (B, D) Methylated primary gastric tumors show weak diffuse IHC staining.*

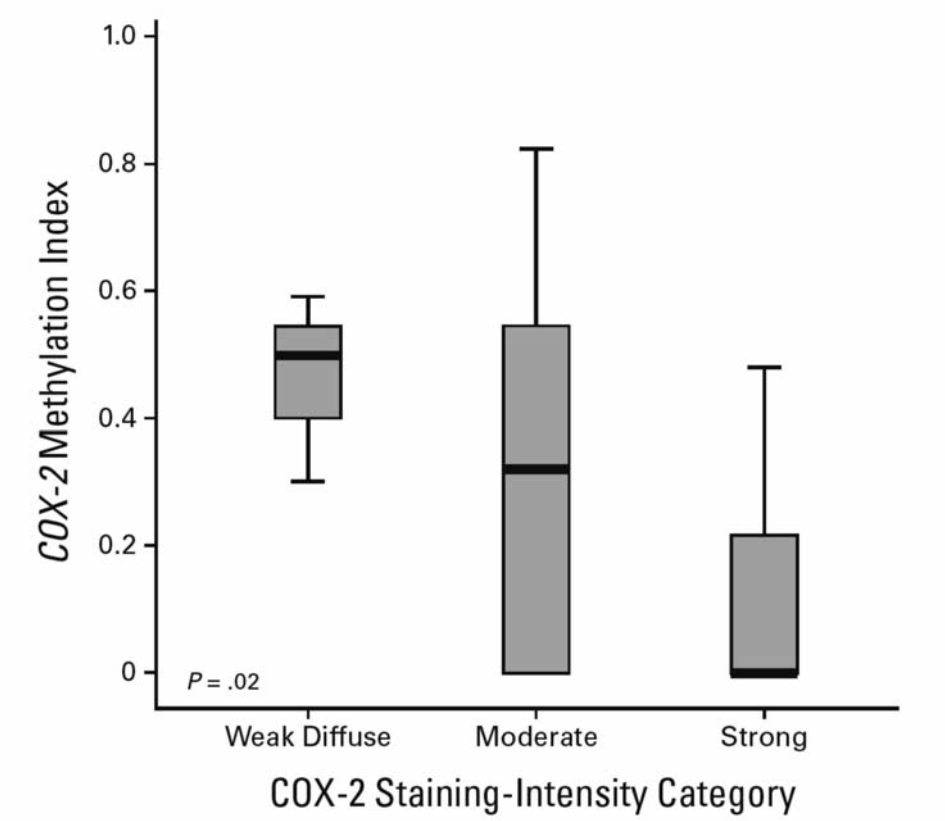

Figure 3. *Boxplots showing gastric primary tumor cyclooxygenase-2 (COX-2) methylation index (y-axis) in relation to COX-2 protein expression categories (x-axis) as assessed by immunohistochemistry in gastric tumor cells.*

COX-2 methylation and clinical outcome

We evaluated gastric cancer patients' clinical prognostic factors between *COX-2* methylated and non-methylated FAMTX trial tumors in a pilot study. *COX-2* methylation status showed no relation to sex, age, Lauren type, T-, N- or TNM-stage, or resectability in this patient group (**table 1**). Univariate analysis of Kaplan Meier survival curves (**figure 4A-B**) demonstrated that *COX-2* methylation status gave significant differences in time to recurrence (TTR) and overall survival (OS). Five patients that did not receive a curative (R0) resection were excluded for the analysis of TTR. Multivariate analysis (**table 2**) showed that methylation of the *COX-2* gene was a favorable independent prognostic factor for TTR and OS. Nodal status showed the strongest predictive value, as expected. The multivariate analysis also showed predictive value for the FAMTX trial randomization arm, indicating improved outcome for patients that received surgery alone.

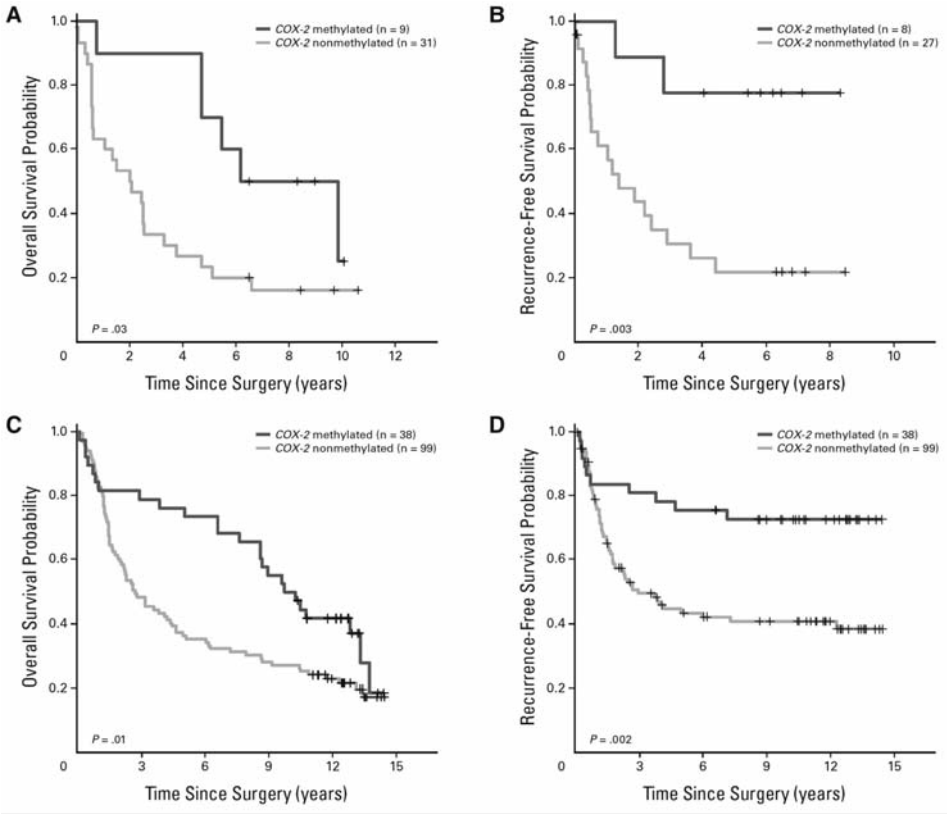

Figure 4. *Kaplan-Meier analysis of survival for gastric cancer patients with primary tumors assessed for methylation status of cyclooxygenase-2 (COX-2). Among 40 fluorouracil, doxorubicin, and methotrexate (FAMTX) trial patients, those with methylated primary tumors had a significantly improved (A) overall survival and (B) longer time to recurrence for 35 patients that could be evaluated for this outcome parameter. (C, D) Represent Kaplan-Meier analysis of 137 D1D2 trial gastric cancer patients.*

Based on the above pilot studies, we validated the utility of *COX-2* methylation status in an independent gastric cancer population that received no neo-adjuvant treatment. To establish sample size we initially performed power calculations using the results of the test-set. Based on these values we calculated 129 patients to be sufficient to obtain significance with an alpha-level of 0.05 and 90% power. Because of expected difficulties in retrospectively analyzing (on average) 12 year old tissue blocks, we added 40% extra patients to account for patients of which no results could be generated. To obtain a homogenous study group we requested blocks of patients without postoperative mortality and that received R0 resection (resection margin microscopically free of tumor). These patients are more likely to be node-negative. An unbiased selection was performed to assure sufficient events for survival analysis. This resulted in that 37% of patients analyzed were node-negative in the

COX-2 Trial Patients						
	FAMTX (n=40)			D1D2 (n=137)		
Variable	**Meth**	**Unmeth**	**P***	**Meth**	**Unmeth**	**P***
No. of patients	9	31		38	99	
Sex						
Female	5	15	.84	19	47	.79
Male	4	16		19	52	
Age, years						
Mean	61.7	60.1	.58†	66.9	67.2	.85†
SD	6.6	9.9		8.7	8.2	
Randomization assignment						
FAMTX surgery/D1 resection	4	15	.84	24	54	.36
Surgery alone/D2 resection	5	16		14	45	
Lauren classification						
Intestinal	6	17	.59	33	73	.34
Diffuse	3	14		4	23	
Mixed	—	—		1	3	
UICC pathologic stage						
IA	2	2	.43‡	15	14	.008‡
IB	2	5		8	23	
II	2	15		7	30	
IIIA	3	4		5	21	
IIIB	0	1		1	6	
IV	0	4		2	5	
UICC nodal status						
N0	4	12	.71‡	23	28	.006‡
N1 (1-6 positive)	5	15		9	52	
N2 (7-15 positive)	0	3		4	16	
N3 (16 positive)	0	1		2	3	
Tumor invasion						
T1	2	2	.68‡	15	24	.02‡
T2	4	19		20	52	
T3	3	10		3	21	
T4	—	—		0	2	
Curative resection						
Yes	8	27	.89	—	—	—
No	1	4		—	—	

Table 1. *Association Among COX-2 Methylation and Clinicopathologic Variables*

Abbreviations: COX-2, cyclooxygenase-2; FAMTX, fluorouracil, doxorubicin, and methotrexate; Meth, methylated COX-2 promoter; Unmeth, nonmethylated COX-2 promoter; UICC, International Union Against Cancer; SD, standard deviation. Correlations by Fisher's exact test; †calculated by t test; ‡calculated by Mann-Whitney U test.

	Time to Recurrence			Overall Survival		
Trial	HR	95% CI	P	HR	95% CI	P
FAMTX						
T stage	—	—	.73	—	—	.59
No. of involved nodes			.01			.04
N0 (0)	1			1		
N1 (1-6)	3.70	1.15 to 11.96	.03	3.66	1.31 to 10.14	.01
N2 (7-15)	5.28	0.53 to 52.91	.16	3.10	0.58 to 16.62	.19
N3 (16)	159.59	6.61 to 3852.78	.002	16.18	1.42 to 184.90	.03
TNM stage	—	—	.60	—	—	.53
Curative resection	NA		NA	—	—	.39
Randomization assignment (surgery alone)	0.23	0.08 to 0.73	.01	0.30	0.12 to 0.71	.008
Intestinal type (Lauren)	—	—	.18	—	—	.09
COX-2 methylated	0.08	0.01 to 0.65	.02	0.37	0.14 to 1.00	.05
D1D2						
T stage	—	—	.16	—	—	.31
No. of involved nodes			<.0001			<.0001
N0 (0)	1			1		
N1 (1-6)	8.67	3.36 to 22.37	<.0001	1.68	1.08 to 2.67	.03
N2 (7-15)	24.25	8.66 to 67.87	<.0001	4.53	2.45 to 8.38	<.0001
N3 (16)	68.91	18.32 to 259.27	<.0001	11.54	4.17 to 31.92	<.0001
TNM stage	—	—	.17	—	—	.38
Randomization assignment (D1 resection)	—	—	.65	—	—	.85
Intestinal type (Lauren classification)	0.57	0.35 to 0.95	.03	0.63	0.42 to 0.93	.02
COX-2 methylated	0.49	0.24 to 0.99	.05	0.62	0.38 to 0.99	.05

Table 2. *Multivariate Analysis of Prognostic Factors As Covariables with COX-2 Methylation Status for Gastric Cancer Disease Outcome*

NOTE. Stepwise Cox regression model.

Abbreviations: COX-2, cyclooxygenase-2; HR, hazard ratio; FAMTX, fluorouracil, doxorubicin, and methotrexate; NA, not applicable.

	Patients Receiving R0 Resection Without Postoperative Mortality From the D1D2 Trial (n=595)				
	Nonanalyzed (n=458)		Analyzed (n=137)		
Factor	No.	%	No.	%	P
Sex					
Female	189	43	66	48	.31
Male	260	57	71	52	
Age, years					
Median	63		66		
SE	0.6		0.7		.001
Random assignment					
D1 resection	247	54	78	57	.54
D2 resection	211	46	59	43	
T stage					
T0	5	1	0	0	.49
T1	131	28	39	28	
T2	213	47	74	53	
T3	99	22	22	17	
T4	10	2	2	2	
N stage					
N0	229	50	51	37	.05
N1 (1-6)	163	36	61	44	
N2 (7-15)	47	10	20	15	
N3 (16)	19	4	5	4	

Table 3. *Comparison of Clinical Characteristics Between Selected and Non- Selected Cases From Curatively Resected Patients of the D1D2 Trial*

*Calculated by Mann-Whitney U test.

validation group (similar as in the test-set). Finally, 178 blocks were processed. Molecular data could be generated of 137 patients. The patients in analysis were than assessed for differences from the non-analyzed patients (**table 3**). As expected, the selected study group showed significant higher proportion of node-positive patients. Older gastric cancer patients are more likely to be node-positive at time of surgery and this explained the higher age of the 137 analyzed patients. Thirty-eight (28%) patients were classified as *COX-2*methylated using the pilot study cut-off (MI=0.24). Mean MI was 0.71 (S.D. 0.29, range 0.26-1.00) in methylated tumors and 0.01 (S.D. 0.04, range 0-0.19) in unmethylated primaries. In this validation group, significant correlation was observed for *COX-2* methylated tumors with lower TNM-stage, node negativity, and lower T-stage (**table 2**). The predictive value of methylation status was confirmed in univariate and multivariate analysis for OS and TTR (**figure 4, table 2**).

Nodal involvement was the strongest prognostic variable and was present in all patients with methylated *COX-2* that relapsed within 1.8 years following surgery. Patients with *COX-2* methylation had improved TTR when only node-negative patients were analyzed (P=0.03). Lauren type, showing borderline significance in the FAMTX-trial, was selected as an independent factor in the larger study group. T-stage was not a prognostic factor and this may be due to the tight assocation with nodal status (P<0.0001). Because of this dependence, TNM-stage was not a significant factor either in multivariate analysis. Trial randomization to extended or limited nodal dissection had no predictive value as shown in the initial trial analyses[24].

Discussion

In gastrointestinal cancers, the role of *COX-2* in tumor-promotion has been shown[34,35]. Numerous stimulatory factors, such as growth factors and cytokines, have been reported to cause overexpression of *COX-2* in cancer[34,36-38]. Studies have suggested methylation as a regulatory mechanism of *COX-2* expression in gastric cancer *in vitro* and in primary tumors[19,33,39-41]. Recently it was reported that C/EBPbeta (CCAAT/enhancer-binding protein beta) transcription factor regulates the expression of endogenous COX-2 in a gastric cancer line model, based on its methylation status[42]. The *COX-2* methylated status may abolish the effect of factors, such as C/EBPbeta, growth factors, or cytokines, due to the inactivation of binding elements at the promoter region. Furthermore, *COX-2* methylation status in our study was independent in multivariate analyses of nodal status which is a highly important predictor of disease recurrence. This independence may be explained by some tumor-enhancing processes intrinsic to gastric adenocarcinoma that increased *COX-2* expression is reported to be associated with. Examples of such processes are angiogenesis, reduced apoptosis and increased inflammation. Also, our data suggests that *COX-2* to be involved in Lauren's histologic classification. In the pilot study, intestinal type tumors correlated to decreased IHC staining intensity (P=0.03), and absence of *COX-2* expression in the intestinal tumor type has been previously reported[20]. In the D1D2 trial patient group diffuse type tumors had a significantly decreased MI compared to intestinal type tumors (P=0.03). Together, methylation of *COX-2* may cause gastric tumor cells to maintain a more differentiated, intestinal organization, and subsequently these tumors may constitute a clinically less aggressive tumor type.

The outcome of both trials, for which patient specimens were used in this study, was negative. The FAMTX trial did not show improved resectability rate of gastric cancer by preoperative chemotherapy, and extended D2 nodal dissection did not reduce recurrence rates, indicating the problem of managing gastric cancer disease. Development of molecular biomarkers for primary gastric cancer tumors may allow for better management strategies. Long-term analyses of the D1D2 trial suggested that node-positive gastric cancer patients may benefit from more extensive nodal dissection at primary tumor surgery[24], and two large randomized trials have now shown the benefit of (neo-)adjuvant treatment for gastric cancer[3,4]. Our study confirms that presence of nodal involvement remains the single most important factor to base treatment decisions upon postoperatively. However, the D1D2 trial data showed that 20% of node negative patients show disease recurrence wit-

hin 3 years after surgery. Biomarkers to select poor-prognosis, node-negative patients that could be potentially useful for stratification in patients receiving adjuvant or neoadjuvant therapy. *COX-2* methylated tumors did show significantly less recurrence in univariate analysis in node-negative patients only, however, our study was underpowered to show independence of *COX-2* methylation status as a predictor of poor prognosis in node-negative patients. The actual advantage of our study for clinical applicability lies in that *COX-2* methylation status is a primary tumor characteristic and therefore can be assessed in standard performed diagnostic tumor biopsy. Primary tumor *COX-2* methylation status therefore may be used as a preoperatively assessable biomarker to tailor more aggressive treatment modalities at time of surgery to gastric cancer patients with poorer prognosis. To date no such biomarkers are preoperatively available in gastric cancer.

DNA is much more stabile in archived tissues as compared to proteins. Our approach to assess gene *COX-2* methylation instead of protein expression by IHC analysis may be more promising as a biomarker in assessment of archival specimens. Furthermore, our study is the first in gastric cancer to show that DNA methylation is an independent prognostic factor predicting a favorable effect on patient outcome by down-regulating the expression of a known tumor-enhancing protein. Our results shift the paradigm that tumor acquired DNA methylation of promoter regions results in adverse outcome in gastric tumors.

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