Methylation of Tumor-Related Genes in Neoadjuvant-Treated Gastric Cancer: Relation to Therapy Response and Clinicopathologic and Molecular Features

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Abstract

Purpose: The objective of this study was to analyze the hypermethylation of tumor-related gene promoters for an association with therapy response and clinicopathologic features of neoadjuvant-treated gastric cancer patients. Furthermore, we analyzed the relationship of promoter hypermethylation with microsatellite instability and loss of heterozygosity (LOH) of the tumors.

Experimental Design: Pretherapeutic biopsies of 61 patients, subsequently treated with cisplatin and 5-fluorouracil, were studied. Methylation analysis of six gene promoters was done using MethyLight technology. Microsatellite analysis was mainly done in previous studies.

Results: The methylation frequencies for the analyzed genes were MGMT, 44%; LOX, 53%; p16, 46%; E-cadherin, 30%; 14-3-3σ, 69%; and HPRT1, 82%. Concordant methylation of more than three genes was found in 46% of the tumors and was inversely correlated with the LOH rate ($P = 9 \times 10^{-5}$) and associated with female gender ($P = 0.049$), nonintestinal type tumors ($P = 0.04$), and a nonproximal tumor location ($P = 0.003$). No statistically significant association between the methylation of a single gene or the concordant methylation of multiple genes was found with response or survival. However, patients with none or only one methylated gene showed a trend for an increase in survival (5-year survival rate, 83% versus 35%; $P = 0.067$).

Conclusion: The highly significant inverse correlation of promoter methylation and LOH rate reflects major alternative molecular pathways in gastric carcinogenesis. Methylation was not statistically significantly associated with the response to cisplatin/5-fluorouracil-based therapy. However, a concordant methylation of more than three genes defines subgroups of gastric cancer with distinct biological and genetic characteristics.

Epigenetic alterations of DNA have been shown to be of high significance in the tumorigenesis of different tumor types (1, 2). Methylation of cytosine residues in the CpG-rich regions, the “CpG-islands,” of gene promoters leads to transcriptional repression of tumor suppressor genes, DNA repair genes, and genes involved in metastasis and invasion. Gene silencing by this promoter hypermethylation is observed in a considerable number of gastric carcinomas and has been detected in both early stages and premalignant lesions. This points to a critical role for epigenetic inactivation mechanisms in the carcinogenic process in the stomach (3–6). The concordant hypermethylation of multiple genes, termed the CpG island methylator phenotype (CIMP), has been described in various tumor types, including both colorectal and gastric carcinomas (7–10). The exact nature of this methylator phenotype, which may reflect a specific type of epigenetic instability, is poorly understood and controversially discussed (11). Several studies have shown that CIMP-positive tumors show a specific genetic profile and are associated with particular clinicopathologic variables (7–10). A prognostic relevance for CIMP after standard therapy or a 5-fluorouracil (5-FU)–based chemotherapy has been reported for different tumor entities, again including gastric carcinoma (12–16).

Gastric carcinoma is characterized by a high mortality rate that is mainly due to the initial diagnosis being made at late/advanced stages. A neoadjuvant chemotherapy, based on 5-FU and cisplatin, is frequently used for the treatment of advanced gastric carcinomas. In a randomized trial, the perioperative treatment resulted in a significantly improved survival compared with surgery alone (17). However, only 20% to 30% of patients respond to this therapy, with the majority undergoing several months of toxic and costly therapy without a survival benefit (18). Thus, there is a pressing need for molecular markers that can be used to predict the individual response to therapy.

The goal of our study was to investigate the occurrence of the hypermethylation of tumor-related genes and to determine if they may be predictors of response and survival of advanced gastric cancer patients treated with a neoadjuvant, cisplatin/
We have investigated if hypermethylation of single tumor and/or therapy-related genes or concordant hypermethylation of multiple genes is associated with patient outcome or other clinical features.

Finally, we analyzed the methylation status for an association between microsatellite instability (MSI) and loss of heterozygosity (LOH), which for the majority of tumors had previously been studied (19, 20). In these previous studies, we had shown a significant association of a high LOH rate with the response to a cisplatin/5-FU–based neoadjuvant chemotherapy (19, 20). Thus, the characterization of epigenetic changes in relation to these other genetic alterations might not only provide some deeper insight into their role for the carcinogenic process in the stomach but might especially be important in the light of a potential application of new epigenetic-based chemotherapeutic strategies for gastric cancer patients.

**Materials and Methods**

**Patients.** Pretherapeutic biopsies of 61 patients with locally advanced gastric cancer (tumor category cT3 or cT4) were analyzed. All patients were treated at the Department of Surgery between 1993 and 2003 with a uniform, combined preoperative chemotherapy containing cisplatin, leucovorin, and 5-FU (PLF-regimen). Procedures for staging, response evaluation, and surgery were according to institutional standard operating procedures, which have been described in detail (21–23) and which did not change during the time of the recruitment of the patients. Selection criteria for the study were the availability of pretherapeutic biopsies and the suitability of material for the isolation of DNA from tumor areas containing at least 50% tumor cells by manual microdissection. In addition, each selected patient must have received 50% of the projected dose of the chemotherapeutic regimen.

Of the 61 patients, 48 (79%) had a complete tumor resection (R0), 10 (16%) had a resection with tumor involved resection margins (R1) after chemotherapy, and 3 (5%) were not resected due to tumor progression. All 61 patients were evaluated clinically for response and all were included in this study (nonewerelosttofollow-up). The median follow-up of the patients was 58.7 months (range, 26.1-106.7 months). The median survival of the patients was 40.6 months (range, 2.4-106.7 months). The median event-free survival (time to progression or time to recurrence) was 27.2 months (range, 3.3-106.7 months). The patient characteristics are included in Table 1.

**Preoperative chemotherapy.** Preoperative therapy consisted of two cycles of combination chemotherapy, each of 49-day duration. On day 1, cisplatin at a dose of 50 mg/m² body surface area was given by i.v. infusion over a period of 1 h. Thereafter, patients received leucovorin (500 mg/m² body surface area) over a period of 2 h, followed by 5-FU (2 g/m² body surface area) over a period of 24 h. Treatment with cisplatin was repeated on days 15 and 29. Infusion of leucovorin and 5-FU was repeated on days 8, 15, 22, and 29 (21, 24). The inclusion and exclusion criteria for chemotherapy were as previously published (19, 24). No adjuvant chemotherapy or radiochemotherapy was applied. Surgical resection of the tumor according to local standards was scheduled 3 to 4 weeks after completion of the chemotherapy (21, 24).

**Response evaluation.** Response evaluation was done histopathologically and clinically as previously described (22, 24, 25). For histopathologic response evaluation, the macroscopically identifiable

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**Table 1. Association of highly methylated tumors and clinicopathologic or molecular features**

<table>
<thead>
<tr>
<th>Total no. patients</th>
<th>MET-H ≥4/6 genes*</th>
<th>MET-M/L &lt;4/6 genes*</th>
<th>P †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total 61 (100)</td>
<td>28 (46)</td>
<td>33 (54)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female 12 (20)</td>
<td>9 (32)</td>
<td>3 (9)</td>
<td>0.049</td>
</tr>
<tr>
<td>Male 49 (80)</td>
<td>19 (68)</td>
<td>30 (92)</td>
<td></td>
</tr>
<tr>
<td>Lauren type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal 29 (48)</td>
<td>9 (32)</td>
<td>20 (61)</td>
<td>0.040</td>
</tr>
<tr>
<td>Nonintestinal 32 (52)</td>
<td>19 (68)</td>
<td>13 (39)</td>
<td></td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 + G2 10 (16)</td>
<td>4 (14)</td>
<td>6 (18)</td>
<td>0.741</td>
</tr>
<tr>
<td>G3 51 (84)</td>
<td>24 (86)</td>
<td>27 (82)</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal third 46 (75)</td>
<td>16 (57)</td>
<td>30 (91)</td>
<td>0.003</td>
</tr>
<tr>
<td>Middle third 10 (16)</td>
<td>7 (25)</td>
<td>3 (9)</td>
<td></td>
</tr>
<tr>
<td>Distal third 2 (3)</td>
<td>2 (7)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Total 3 (5)</td>
<td>3 (11)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>MSI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-H 6 (10)</td>
<td>1 (3)</td>
<td>5 (15)</td>
<td>0.143</td>
</tr>
<tr>
<td>MSI-L 12 (20)</td>
<td>8 (29)</td>
<td>4 (12)</td>
<td></td>
</tr>
<tr>
<td>MSS 43 (70)</td>
<td>19 (68)</td>
<td>24 (73)</td>
<td></td>
</tr>
<tr>
<td>FAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAL-H 55 ‡ (100)</td>
<td>27 (49)</td>
<td>28 (51)</td>
<td>9 x 10^-5</td>
</tr>
<tr>
<td>FAL-M 11 (20)</td>
<td>1 (4)</td>
<td>10 (36)</td>
<td></td>
</tr>
<tr>
<td>FAL-L 22 (40)</td>
<td>8 (30)</td>
<td>14 (50)</td>
<td></td>
</tr>
<tr>
<td>Mean age (range), y</td>
<td>55.13 (29.8-72.4)</td>
<td>55.10 (40.3-71.5)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

* Number of methylated genes / analyzed genes per tumor.
† Fisher’s exact test (two sided) or Mann-Whitney test.
‡ MSI-H tumors (n = 6) were not evaluated for FAL.
tumor bed of resected tumors was histologically completely examined. Those patients who had <10% residual tumor in the tumor bed area were classified as responders. All other patients, including patients with clinical progression, were classified as nonresponders (24, 25).

Clinical response evaluation was done by measuring the size of the primary tumor by computed tomography scan, endoluminal ultrasound, and endoscopy and was defined as previously published (24, 25).

For the analysis of the association between methylation status and response, only patients with a congruent evaluation by both methods (n = 54) were used as previously described (25). This included 15 (28%) responders and 39 (72%) nonresponders.

Patient survival was significantly associated with both clinical and histopathologic responses (P < 0.001). Analysis of the methylation status in association with survival was done for all 61 patients.

The study protocol was approved by the local ethics committee and informed consent was obtained according to institutional regulations.

**Gene selection.** The following six genes involved in different molecular pathways were analyzed: p16, 14-3-3σ (cell cycle), O6-methylguanine-DNA methyltransferase (MGMT; DNA repair), E-cadherin, hylx exodise (LOX; cell adhesion), and HPP1. A prerequisite for the selection of these genes was that promoter hypermethylation has previously been established as a mechanism for the transcriptional inactivation of the genes in gastric cancer. Furthermore, the reported methylation frequencies were required to be preferably between 25% and 75% because genes with a very low or a very high methylation frequency are not useful in distinguishing between groups (26–31). Finally, functional aspects were considered. Thus, the genes involved in cell cycle control (i.e., p16 and 14-3-3σ) may also play a direct role for chemotherapeutic efficacy, and a relation to the effect of cisplatin has been reported for the E-cadherin and MGMT genes, making them additionally attractive candidates to test for a potential role in therapy response (32, 33).

**DNA extraction from archival tissues.** DNA from gastric cancer specimens was extracted from formalin-fixed, paraffin-embedded tissue. Defined areas having >50% of tumor cells were manually microdissected and DNA was isolated by proteinase K digestion and phenol-chloroform extraction according to standard procedures (34).

**Sodium bisulfite treatment of DNA.** Treatment of the DNA with sodium bisulfite was done essentially as described (35). Genomic DNA (up to 1 μg) in a volume of 50 μL Tris-EDTA buffer was denatured at 95°C for 10 min, followed by incubation with 5.5 μL of 3 mol/L NaOH for 20 min at 40°C and 3 min at 95°C. Samples were put on ice and treated with 500 μL of freshly prepared 2.8 mol/L sodium bisulfite/2.5 mol/L hydrochloric solution for 3 h at 55°C. DNA was purified using the Wizard DNA clean-up kit (Promega Corp.) according to the manufacturer’s instructions. DNA was eluted with 100 μL of Tris-EDTA buffer, desulfonated with 11 μL of 3 mol/L NaOH for 20 min at 40°C, precipitated with ethanol, and resuspended in 75 μL of distilled water.

**MethyLight analysis.** After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight technique as described (36) using the ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems, Inc.). Published primer and probe systems were used or were designed specifically for fully methylated bisulfite-converted DNA using the primer express software (Applied Biosystems). Primer and probe sequences, as well as reaction conditions, are summarized in Table 2. To normalize for the input of DNA, a region of the MYOD1 gene lacking bisulfite-sensitive CpG islands was used (36). SssI-treated human lymphocyte DNA was used as a fully methylated positive control. PCR products were cloned and sequenced, and DNA of positive clones was used as internal positive

### Table 2. Primer and probe sequences of the MethyLight systems

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer and probe</th>
<th>Sequence 5’-3’</th>
<th>Size of PCR product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MYOD1</strong></td>
<td><strong>Forward</strong></td>
<td>CCAACTCCAAATCCCCCTCTAT</td>
<td>107</td>
<td>(36)</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>GTTTTTTTAGGGAGGTAGTTGTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Probe</strong></td>
<td>TCCCTCTTATCTCTAAATCCACCTAATACCTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>14-3-3σ</strong></td>
<td><strong>Forward</strong></td>
<td>GAAGGTGATAAGTTGAGTAGGGTGCGAC</td>
<td>117</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>AACTACTAAAACAAATATCTGCTTCTTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>E-Cadherin</strong></td>
<td>CTCGCCCCTTCCTCCAGCAGGG</td>
<td>70</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>AAATTGTTAGGGTTAGTACCGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Probe</strong></td>
<td>TCCCAAAAACAAAGAATACCGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>HPP1</strong></td>
<td>GTTATCGTCGCTGTTTTGTTGTC</td>
<td>87</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>GACTTCCGAAATAAACAACACATCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>** Probe**</td>
<td>CCGAAACAACGGAATCAACTAAAATCCCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LOX</strong></td>
<td><strong>Forward</strong></td>
<td>GAATAAAAGATTTGAGGGGGCGT</td>
<td>122</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>GGGACATCTCGAGAGAGGAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Probe</strong></td>
<td>CACGTTTACAAATAATACACCAACCAGTCTAACCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MGMT</strong></td>
<td><strong>Forward</strong></td>
<td>CGATTATCACTAAACAACACGGGG</td>
<td>122</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>GTATTCTTTTTGCGGAGCGAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Probe</strong></td>
<td>AATCGTCCGACATACCCCGTTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p16</strong></td>
<td><strong>Forward</strong></td>
<td>GGGAGGAGTAGGATGCCGGG</td>
<td>84</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>AAACCAAATACCCGAAATCTCCATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Probe</strong></td>
<td>TACCTCCCGGGCAGGCACCTCCAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Primer and probe concentrations were for Myod1, 300 nmol/L primer/50 nmol/L probe; 14-3-3σ, 600 nmol/L primer/200 nmol/L probe; E-cadherin, 600 nmol/L primer/200 nmol/L probe; HPP1, 600 nmol/L primer/100 nmol/L probe; LOX, 600 nmol/L primer/100 nmol/L probe; MGMT, 600 nmol/L primer/100 nmol/L probe; and p16, 600 nmol/L primer/75 nmol/L probe.
control. Human sperm DNA or DNA from lymphocytes was used as the unmethylated negative control.

PCR was done in duplicate in a final volume of 30 μL with the TaqMan Universal PCR Master Mix without uracil-N-glycosylase (Applied Biosystems) using 5 μL of bisulfite-treated DNA as template. Cycling conditions were 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Samples with no detectable methylation signal using 45 cycles but with successful amplification of MYOD1 were considered to be nonmethylated. Results with SD >30% were repeated. Relative promoter methylation levels were determined by the standard curve method, defining SsI-treated human lymphocytic DNA as 100% methylation ratio.

The frequency of methylation of the genes was determined by choosing a specific cutoff value for the respective genes. As the determination of cutoff values is not standardized and the detection of methylation may depend on the amplification efficiency of the reaction of the individual genes, and may for some cases be influenced by patient specific characteristics, we defined methylation relative to the methylation signal detected in nontumorous gastric epithelial tissues. Thus, methylation was defined as positive if the percent methylation ratio was above the median percent methylation ratio of the respective gene in an analysis of 10 nontumorous gastric epithelial tissues from randomly selected patients. The cutoff values were as follows: 0.01 for LOX, p16, MGMT; 2.23 for HPP1; 3.45 for E-cadherin; and 23.49 for 14-3-3ζ.

Tumors were grouped according to the number of methylated genes per six analyzed genes found per tumor. There is no standardized definition of highly methylated tumors that most likely represent the CIMP phenotype. We applied the following classification systems: we categorized the tumors into a high methylation group (MET-H) with tumors showing ≥4 methylated genes, a medium methylation group (MET-M) with tumors showing 2 to 3 methylated genes, and a low methylation group (MET-L) with tumors showing 0 to 1 methylated genes. We analyzed (a) the MET-H versus the MET-M/L group and (b) the MET-H/M group versus the MET-L group for an association with the clinicopathologic features, therapy response, and molecular variables.

Microsatellite analysis. Of the 61 tumors analyzed for promoter hypermethylation in the present study, 34 had previously been characterized by microsatellite analysis (19, 20). To allow for a comparison of promoter hypermethylation with MSI and LOH in the whole study group, microsatellite analysis was done for the remaining 27 cases with the same 11 microsatellite markers and protocol, using fluorescence labeled primers and separation and detection by an automated sequencing system (ABI 377, Perkin-Elmer) as published (19, 20).

Instability in ≥40% of the analyzed markers was defined as MSI-H, instability in <40% as MSI-L, and no instability as microsatellite stable (MSS).

LOH was defined if the allele peak ratio was <60%, representing a signal reduction of one allele of at least 40% (19, 20). Fractional allelic loss (FAL) was defined as the ratio of the number of chromosomal sites showing LOH divided by the number of informative chromosomal sites for each case. The tumors were categorized as low (0-0.25), medium (0.25-0.5), and high FAL (0.5) as described (19). Tumors with MSI-H were excluded from evaluation for FAL.

Statistical analysis. Fisher’s exact test (two sided) was used to compare for the relative frequencies between different groups. Differences between survival were evaluated using the log-rank test. All survival data were calculated from the start of chemotherapy to the date of death or most recent follow-up. The Mann-Whitney test was used to compare metric variables in two groups. P < 0.05 was considered to be statistically significant and P < 0.1 was considered as trend. Statistical analysis was done using the SPSS software (SPSS, Inc.).

Results

Methylation frequencies. Among the 61 gastric carcinomas, promoter methylation of the six genes analyzed was detected at the following frequencies: 18 (30%) for E-cadherin, 27 (44%) for MGMT, 28 (46%) for p16, 32 (53%) for LOX, 42 (69%) for 14-3-3ζ, and 50 (82%) for HPP1.

Partition of the tumors into a high methylation group (MET-H; ≥4/6 methylated genes) and a medium/low methylation group (MET-M/L; <4/6 methylated genes) revealed that 28 of 61 (46%) tumors belonged to the MET-H group and 33 of the 61 (54%) belonged to the MET-M/L group.

Categorization of the tumors into a high/medium methylation group (MET-H/M; ≥2/6 methylated genes) and a low methylation group (MET-L; 0-1/6 methylated genes) revealed that, overall, 52 of 61 (85%) tumors belonged to the MET-H/M group and 9 of 61 (15%) tumors belonged to the MET-L group.

Concordant methylation and correlation with MSI and LOH. Among the 61 tumors included in this study, 6 (10%) tumors were MSI-H, 12 (20%) were MSI-L, and 43 (70%) were MSS. The degree of MSI was not correlated with the methylation of multiple genes, comparing the MET-H group with the MET-M/L group and (MET-M/L; <4/6 methylated genes) revealed that 28 of 61 (46%) tumors belonged to the MET-H group and 33 of the 61 (54%) belonged to the MET-M/L group.

Categorization of the tumors into a high/medium methylation group (MET-H/M; ≥2/6 methylated genes) and a low methylation group (MET-L; 0-1/6 methylated genes) revealed that, overall, 52 of 61 (85%) tumors belonged to the MET-H/M group and 9 of 61 (15%) tumors belonged to the MET-L group.

Concordant methylation and correlation with MSI and LOH. Among the 61 tumors included in this study, 6 (10%) tumors were MSI-H, 12 (20%) were MSI-L, and 43 (70%) were MSS. The degree of MSI was not correlated with the methylation of multiple genes, comparing the MET-H group
versus the MET-M/L group or comparing the MET-H/M group versus the MET-L group (Table 1 and data not shown).

Among the 55 tumors that were evaluated for LOH, 11 (20%) showed LOH at >50% of the informative chromosomal sites and were classified as FAL-H tumors; 22 (40%) showed LOH between 25% and 50% of the informative chromosomal sites and were classified as FAL-M tumors; and 22 (40%) showed LOH at <25% of the chromosomal sites and were classified as FAL-L. Analyzing the FAL rate for an association with the methylation rate of the tumors revealed a highly statistically significant association of MET-H tumors and low FAL levels and vice versa ($P = 9 \times 10^{-5}$). The data are included in Table 1 and shown in Fig. 1.

Methylation and correlation with clinicopathologic variables. Correlation of concordant methylation of multiple genes with clinicopathologic variables, such as age, gender, Lauren classification, tumor grading, and tumor location, revealed a statistically significant association between MET-H tumors and female gender ($P = 0.049$), nonintestinal type tumors ($P = 0.040$), and nonproximal tumor location ($P = 0.003$). These data are summarized in Table 1.

Considering methylation of the single genes, the following statistically significant correlations were found: methylation of the $LOX$ gene was associated with nonintestinal type tumors ($P = 0.011$), worse tumor differentiation ($P = 0.037$), and nonproximal tumor location ($P = 0.010$); methylation of the $14-3-3\sigma$ gene was associated with female gender ($P = 0.012$); and methylation of the $HPP1$ gene was associated with age ($P = 0.03$).

Methylation and correlation with response and survival. The analysis of the number of methylated genes per tumor in relation to therapy response revealed a higher methylation frequency among the nonresponding patients (Fig. 2). Comparing the patients with tumors of the MET-H group versus the MET-M/L group showed that 19 of 39 (49%) of the non-responding and 4 of 15 (27%) of the responding patients were MET-H tumors ($P = 0.22$). Comparing the patients with tumors of the MET-L group versus the MET-H/M group showed that 4 of 39 (10%) among the nonresponding and 4 of 15 (27%) of the responding patients were MET-L ($P = 0.20$). These differences, however, were not statistically significant (Table 3).

A trend toward longer survival was observed for patients with MET-L tumors. The median overall and the median event-free survival time in the MET-L group were 87.87 months or were not reached, respectively, compared with 44.0 and 22.2 months, respectively, in the MET-H/M group ($P = 0.191$ and $P = 0.097$, log-rank test). Using the 5-year survival rate as an end point, a statistical trend for an increase in survival was also found, as 83% of the patients with MET-L tumors were still alive compared with 35% with MET-H/M tumors ($P = 0.067$). All data are summarized in Table 3.

Evaluation of methylation of the single genes revealed a trend for an association with therapy response for the $LOX$ gene, with 4 of 15 (27%) responders and 23 of 39 (59%) nonresponders showing methylation in their tumors ($P = 0.067$). No associations with survival were found (Table 4).

Discussion

In this study, we have analyzed promoter hypermethylation of six gastric cancer related–genes in 61 pretherapeutic biopsies of patients, who subsequently received a neoadjuvant treatment based on cisplatin and 5-FU. As recent studies emphasized the existence of a so-called CIMP as a distinct molecular subgroup of gastrointestinal tumors (7, 10, 13, 16, 27, 37), we were particularly interested if the concordant methylation of multiple genes was associated with response and/or survival of the patients.

For colorectal cancer, an optimal marker panel for the characterization of CIMP has recently been determined (7). Because there is no standardized criteria for the definition of CIMP in gastric cancer, we analyzed the concordant methylation of multiple genes using two different classification systems.

Overall, we found that 46% of the tumors had concurrent hypermethylation of at least four of the six analyzed genes. This is similar to previous studies reporting a concurrent hypermethylation of multiple genes in 31% to 41% of gastric carcinomas (3, 13, 37). In our study, these tumors showed distinct molecular and clinicopathologic features. The most interesting of which was the highly significant inverse correlation between the methylation rate and the rate of LOH (FAL value; $P = 9 \times 10^{-5}$). This suggests that epigenetic instability, reflected by the concordant promoter hypermethylation of multiple genes, and chromosomal instability, reflected by a high FAL value or high LOH rate, represent pathways of
genetic alterations driving distinct carcinogenic pathways in gastric cancer. Similar results have recently been reported for colorectal carcinomas (38). In gastric cancer, analyzing the inactivation mechanisms of the \( \text{E-cadherin} \) gene, a mutual exclusivity between LOH and hypermethylation has been reported, which is essentially in line with our results (39). Altogether, this strongly supports the existence of two independent mechanisms of genetic and epigenetic instability in gastrointestinal cancer.

Considering the methylation rate in relation to response to therapy, we observed tumors with concurrent methylation of multiple genes more frequently among nonresponding patients, although the differences did not reach statistical significance in either of the two classification systems tested \( (P = 0.2 \text{ for both}) \).

In previous studies, we have shown that there is a statistically significant relationship between response to a cisplatin/5-FU-based chemotherapy and the FAL rate because patients showing high FAL values in their tumors were more frequently found among responding patients \( (19, 20) \). This finding holds true also for the tumors included in the present study \( (P = 2 \times 10^{-5}; \text{ data not shown}) \). Taken together with the highly inverse correlation observed between the FAL and methylation rates, this raises the important question if an epigenetic-based chemotherapy may represent an alternative treatment.

With respect to survival, a trend for an increase in the event-free survival time and in the 5-year survival rate was found for the group of patients showing none or one methylated gene in their tumors \( (P = 0.097 \text{ and } P = 0.067, \text{ respectively}) \). At first, this contradicts other studies reporting an association between the concordant methylation of multiple genes and better survival after surgery for gastric cancer \( (13, 16) \). However, it has to be stressed that the survival rates for treatment by surgery alone are not comparable with those for neoadjuvant treatment and surgery used in our study. Furthermore, in the study by An et al. \( (13) \), methylation of the \( \text{MLH1} \) gene was included in the analysis. This favors the inclusion of microsatellite unstable tumors in the group of highly methylated tumors. Because patients with microsatellite unstable tumors have been shown to have a better prognosis in gastric and colorectal cancers \( (44, 45) \), this may lead to confounding results about the prognostic significance of methylation of multiple genes. Furthermore, as the number of patients included in our study is relatively small, the prognostic significance of promoter hypermethylation and survival has to be considered with care and an extended analysis in a larger number of cases is needed to draw firm conclusions.

### Table 3. Methylation status and association with therapy response and survival

<table>
<thead>
<tr>
<th>Methylation status</th>
<th>No. methylated tumors</th>
<th>Overall survival</th>
<th>5-y survival</th>
<th>Event-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responder, ( n = 15 ) (%)</td>
<td>Nonresponder, ( n = 39 ) (%)</td>
<td>P*</td>
<td>Median (mo)</td>
</tr>
<tr>
<td>MET-H: ≥4/6 ‡</td>
<td>4 (27)</td>
<td>19 (45)</td>
<td>0.2</td>
<td>Not reached</td>
</tr>
<tr>
<td>MET-M/L: ≤4/6 ‡</td>
<td>11 (73)</td>
<td>20 (51)</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>MET-H/M: ≥2/6 ‡</td>
<td>11 (73)</td>
<td>35 (90)</td>
<td>0.2</td>
<td>44.00</td>
</tr>
<tr>
<td>MET-L: &lt;2/6 ‡</td>
<td>4 (27)</td>
<td>10 (25)</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

*Fisher’s exact test (two sided).
† Log-rank test.
‡ Number of methylated genes / analyzed genes per tumor.
In our study, we did not observe a correlation between concordant methylation of multiple genes and MSI, which has previously been described for gastric cancer by others (29). This controversial result may also be related to the fact that we have not included in our study the MLH1 gene, which is frequently inactivated by promoter hypermethylation in sporadic gastric and colorectal carcinomas showing high MSI (3, 7, 29).

With respect to the association between concurrent methylation of multiple genes and clinicopathologic variables, we found a significant association of the high methylation group and nonintestinal type tumors (P = 0.04). This result is consistent with recent findings that indicate different pathways of gastric carcinogenesis. The so-called “methylator” phenotype is preferentially associated with diffuse type tumors, whereas the more mutational and genomic unstable phenotype is preferentially associated with intestinal type gastric tumors (27, 46). Similar to results described for colorectal carcinomas (7), we found an association of highly methylated tumors with female gender (P = 0.049) and tumor location (P = 0.003), but the reasons are poorly understood.

Considering methylation of the single genes, the observed methylation frequencies ranged from 30% to 82% and were essentially in line with previously published data for gastric cancer (26–30). Interestingly, for the LOX gene, significant associations were found between promoter methylation and nonintestinal type tumors (P = 0.011), worse tumor differentiation (P = 0.037), and nonproximal tumor location (P = 0.012), and a trend was observed for an association with nonresponse (P = 0.067). A correlation of methylation of the LOX gene with the histopathologic type may be related to one of the functions of LOX as an extracellular enzyme that initiates covalent cross-linking of collagens and elastins. Loss of this function might facilitate spreading of tumor cells, which is characteristic of diffuse type gastric cancer. LOX has been described as a tumor suppressor gene and inactivation by methylation and LOH has been shown in gastric cancer (30). Our finding of a preferential methylation of the LOX gene in diffuse type gastric cancer is in line with a previous report (5) and supports a specific role of LOX as a diffuse type gastric cancer tumor suppressor gene.

In summary, we have shown a relative high frequency of concordant methylation of multiple genes in pretherapeutic gastric cancer biopsies. Although methylation of the analyzed genes was not statistically significantly associated with a response to a cisplatin/5-FU–based therapy in this study, we showed that tumors with more than three methylated genes define subgroups with distinct biological and genetic characteristics. In particular, the highly significant inverse correlation between the methylation and LOH rate indicates major alternative molecular pathways in gastric carcinogenesis. This may be of clinical relevance for a potential application of an epigenetic-based chemotherapy in gastric cancer.

Acknowledgments

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Rudolf Napieralski, Katja Ott, Markus Kremer, et al.


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