

Methylation of Serum DNA Is an Independent Prognostic Marker in Colorectal Cancer

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Abstract **Purpose:** Aberrant CpG island hypermethylation is a feature of a subgroup of colorectal cancers, which can be detected in the serum of affected patients. This study was designed to identify methylation targets with prognostic significance in the serum of patients with colorectal cancer. **Experimental Design:** In a gene evaluation set consisting of sera from 24 patients with local colorectal cancers, 14 with metastasized disease, and 20 healthy controls, the genes *HPP1/TPEF*, *HLTF*, and *hMLH1* were identified as potential serum DNA methylation markers. These genes were further analyzed in a test set of sera of 104 patients with colorectal cancer. **Results:** Methylation of *HLTF*, *HPP1/TPEF*, and *hMLH1* was found to be significantly correlated with tumor size, and methylation of *HLTF* and *HPP1/TPEF* was significantly associated with metastatic disease and tumor stage. Moreover, methylation of *HPP1/TPEF* was also associated with serum carcinoembryonic antigen. The prognostic relevance of methylation of these genes was tested in pretherapeutic sera of 77 patients with known follow-up. Patients with methylation of *HPP1/TPEF* or *HLTF* were found to have unfavorable prognosis ($P = 0.001$ and 0.008). In contrast, serum methylation of *hMLH1* was not associated with a higher risk of death. Multivariate analysis showed methylated *HPP1* and/or *HLTF* serum DNA to be independently associated with poor outcome and a relative risk of death of 3.4 (95% confidence interval, 1.4–8.1; $P = 0.007$). **Conclusions:** These data show that the methylation status of specific genes in the serum of patients with colorectal cancer has the potential to become a pretherapeutic predictor of outcome.

Aberrant hypermethylation of DNA is a common finding in human cancer and is found early during carcinogenesis (1). Targets of methylation are CpG islands, which are genetic regions within the 5' untranslated regulatory sequences and first exons of genes containing >500 bp with a guanine plus cytosine content >55% (2). About 40% of mammalian genes contain CpG islands within their promoters and the consequence of methylation is transcriptional silencing. The first gene showed to be a target of CpG island hypermethylation in sporadic tumors was the *CALCA* gene (3). Since then, DNA methylation has been shown to affect many genes in various cancers (e.g., *hMLH1* in colon, *CDH1* in stomach, *VHL* in kidney, *BRCA1* in

breast, and *p16^{INK4A}* in colon, lung, and breast; see ref. 4 for review).

DNA hypermethylation is thought to be a nonrandom process with specific genes being methylated in specific tumors (5). In colorectal cancer which is the second most frequent cancer and one of the leading causes of cancer-related death (6), DNA methylation has been studied extensively and many genes specifically affected by CpG methylation have been identified, including *hyperplastic polyposis 1/transmembrane protein containing epidermal growth factor and follistatin domains (HPP1/TPEF)*, *adenomatous polyposis coli (APC)*, *hypermethylated in cancer 1 (HIC1)*, and *helicase-like transcription factor (HLTF)*; see ref. 7 for review). The phenomenon of simultaneous and intense hypermethylation of multiple genes has been termed the "CpG island methylator phenotype" (CIMP). CIMP was first described based on the finding of concordant methylation events in a subset of colorectal tumors (8), and CIMP⁺ cases have been found to be more common in women, to occur more frequently in the proximal than in the distal colon, as well as to present with a poorly differentiated histology and a distinct profile of genetic abnormalities (9–12). The presence of simultaneous methylation of multiple genes, a hallmark of CIMP, has been found to be associated with poor prognosis in many malignant tumors (9). Recently, a panel of five methylation markers has been proposed to identify CIMP with high sensitivity and specificity (13). In colon, CIMP⁺ as determined by different methylation marker panels has also been shown to be associated with a worse outcome (14) and to have independent

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Received 5/25/06; revised 8/3/06; accepted 8/22/06.

Grant support: Stiftungsfond der Medizinischen Fakultät.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

This work contains parts of the doctoral thesis of M. Wallner.

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doi:10.1158/1078-0432.CCR-06-1264

predictive value for the survival benefit from adjuvant chemotherapy (15). Hypermethylation of the gene *p16*, *MYOD1*, or *ID4* is associated with unfavorable prognosis (16–18).

Methylated DNA derived from primary colorectal cancers cannot only be detected in the tumor tissue itself but also in serum (19–23) and stool of corresponding patients (24–26). Detection of tumor-derived methylated DNA in remote medium may be useful for screening, determination of prognosis, and monitoring therapy. Thus far, little data are available on the prognostic relevance of methylated DNA in the serum of patients with colorectal cancers. We studied the prognostic potential of DNA hypermethylation in pretherapeutic sera of colorectal cancer patients. Three of 12 initially evaluated genes were included in the prognostic analysis. Hypermethylation of the promoters of the genes *HPP1/TPEF* and *HLTF* alone or in combination was found to be an independent prognostic factor in colorectal cancer.

Materials and Methods

Patients and serum samples. The gene evaluation set consisted of 38 patients with newly diagnosed sporadic colorectal cancer, of which

Table 1. Characteristics of the gene evaluation and test sets

Characteristics	Gene evaluation set (n = 38), %	Test set (n = 104), %	P*
Gender			0.117
Male	68	56	
Female	32	44	
Size of tumor [†]			0.631
T ₁	5	8	
T ₂	5	12	
T ₃	69	68	
T ₄	21	12	
Nodal status [†]			0.912
N ₀	40	52	
N ₁	60	48	
Distant metastases [†]			0.675
M ₀	63	83	
M ₁	37	17	
Localization ^{††}			0.739
Right [§]	38	24	
Left	62	76	
Colon	62	58	0.739
Rectum	38	42	
Grading			0.504
G1	3	3	
G2	44	52	
G3 [¶]	53	45	
UICC stage			0.196
I	10	14	
II	21	33	
III	32	36	
IV	37	17	

*P values for the comparison of numbers of patients were calculated by means of the χ^2 test.

[†]For one patient of the test set, tumor localization and information on TNM staging was not available.

^{††}For one patient of the gene evaluation set, tumor localization was unknown.

[§]Right colon includes cecum through transverse colon.

^{||}G3 left colon includes descending colon through rectum.

[¶]G3 includes all cases classified as G3 or as G2-G3.

Table 2. Frequency of methylated genes in serum DNA of the gene evaluation set

Gene	Healthy controls (n = 20)	Local disease (n = 24)	Metastasized disease (n = 14)
<i>APC</i>	20	21	43
<i>HIC1</i>	10	4	14
<i>HLTF</i>	0	17	50
<i>hMLH1</i>	0	4	43
<i>HPP1</i>	0	13	36
<i>p16</i>	60	25	64
<i>RASSF1A</i>	10	4	0
<i>SFRP1</i>	10	17	0
<i>SFRP2</i>	0	4	7
<i>SFRP4</i>	5	13	0
<i>SFRP5</i>	5	4	14
<i>WT1</i>	15	4	0

NOTE: Values in table expressed as % positive.

24 were M₀ (age range, 34-87 years; mean, 67 years) and 14 were M₁ (age range, 33-72 years; mean, 62 years), and 20 controls who received a colonoscopy screening and were found to have a normal colonoscopy and who did not have any known diagnosis of malignant disease (age range, 23-81 years; mean, 58 years). There was no statistical difference between the mean ages of controls and M₀ cases ($P = 0.282$), nor between M₀ and M₁ cases ($P = 0.263$) and not between controls and M₁ cases ($P = 0.261$). The test set consisted of 104 patients diagnosed with colorectal cancer in the years 1991 to 1997 who were operated either at the University Hospital Grosshadern of the University of Munich or the Maria-Theresia Clinic in Munich (age range, 33-92 years; mean, 68 years). There was no statistical difference between the mean ages of patients in the gene evaluation and the test sets ($P = 0.097$). For 77 patients, a 5-year follow-up was available. Characteristics of gene evaluation and test sets are shown in Table 1. In each case, serum was drawn before any therapeutic intervention. The blood was centrifuged at 3000 × g for 10 min at room temperature, and aliquots were stored at -80°C. The study was approved by the ethical committee of the Medical Faculty of the University of Munich.

DNA isolation and bisulfite conversion. Genomic DNA from 1 mL of each serum sample was isolated using the QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sodium bisulfite conversion of genomic DNA was done as described previously (25). After ethanol precipitation, bisulfite-treated DNA was resuspended in 30 μ L Tris-HCl [1 mmol/L (pH 8.0)], aliquoted, and stored at -80°C.

Analysis of DNA methylation. Bisulfite-treated DNA was analyzed by a fluorescence-based, real-time PCR assay, described previously as MethyLight (27, 28). Briefly, two sets of primers and probes, designed specifically to bind to bisulfite-converted DNA, were used (information on sequences of primers and probes is listed in Supplementary Data); one set of primers for every methylated target to be analyzed and a pair

Table 3. Distribution of PMR values in serum of 141 patients with colorectal cancer

PMR	HLTF			HPP1			hMLH1		
	I+II	III	IV	I+II	III	IV	I+II	III	IV
0	50	41	16	58	45	17	42	32	19
0-5	3	2	4	3	2	6	11	13	7
5-25	2	5	2	0	2	4	1	3	1
>25	6	1	9	0	0	4	7	1	4

of primers for the reference gene, β -actin (*ACTB*), to control for DNA amplification and normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed by separately amplifying completely methylated and unmethylated human control DNA (Chemicon, Temecula, CA) with each set of primers and probes. PCRs were done in 20 μ L volumes containing 1 \times PCR buffer (Qiagen), 1.25 mmol/L MgCl₂, 250 μ mol/L deoxynucleotide triphosphate mixture, 90 mmol/L of each primer, 25 mmol/L of each probe, 1 \times Q-Solution (Qiagen), 2 μ L bisulfite-treated DNA, and 0.05 units/ μ L Taq DNA polymerase (HotStar Taq, Qiagen). PCRs were done in a Rotor-Gene RG 3000 (Corbett Research, Sidney, New South Wales, Australia) using the following conditions: 95°C for 900 s followed by 40 cycles of 94°C for 30 s, 60°C for 120 s, and 84°C for 20 s. The percentage of methylated reference at a specific locus was calculated as described previously (29) by dividing the gene/actin ratio of a sample by the gene/actin ratio of fully methylated DNA and multiplying by 100. A gene was deemed methylated if the percentage of the fully methylated reference value was >0. The mean *ACTB* value of the samples collected between 1991 and 1994 was 39.5 \pm 115.8 and the mean value of the samples collected between 1995 to 1997 was 45.8 \pm 79.8 (P = 0.396), indicating that storage of serum samples over longer periods did not result in a significant loss of amplifiable DNA.

Determination of carcinoembryonic antigen values. Carcinoembryonic antigen (CEA) was quantified using a microparticle immunoenzymometric assay (AxSYM, Abbott Laboratories, Chicago, IL).

Statistical analysis. All statistical analyses were done using SPSS software versions 12.0 and 13.0 (SPSS, Inc., Chicago, IL). Pearson's χ^2 test was used to explore associations between clinicopathologic features. Overall survival was calculated from the date of diagnosis of the primary tumor to the date of death or end of follow-up of 5 years. Overall survival curves were calculated with the Kaplan-Meier method. Univariate analysis of overall survival according to clinicopathologic

data and gene methylation status was done using log-rank tests. Cox proportional hazards analysis was used to estimate the prognostic effect of methylated genes, controlling for the classic risk factors tumor size, lymph node status, presence of distant metastases, and age at diagnosis. Two-sided P values < 0.05 were considered as significant.

Results

Identification of appropriate genes in a gene evaluation set. CpG islands of promoters of 12 different genes, which have been reported to be hypermethylated in primary colorectal cancers in various frequencies ranging from 10% to 80% but not in normal colon, were analyzed in the sera of 24 patients with local colorectal cancers [International Union Against Cancer (UICC) I-III], 14 patients with metastasized disease (UICC IV), and 20 healthy controls. An overview of the frequencies of gene methylation in the investigated serum samples is given in Table 2. For further analysis, genes were chosen which were unmethylated in \geq 90% of serum samples from healthy controls, methylated in at least 30% of patients with metastasized colorectal cancer, and more frequently methylated in serum of patients with metastasized than in serum of patients with local disease. Only three genes met these criteria: *HPP1*, *HLTF*, and *hMLH1*. The distribution of percentage of methylated reference of these three genes in the gene evaluation and test sets is shown in Table 3. The best cutoff value determined by the receiver operating characteristic curve was found to be 0.5 for *HPP1* and *HLTF* and 2.0 for *hMLH1*. All of the following analyses were done quantitatively

Table 4. Frequency of methylated genes according to clinicopathologic features

Characteristics	No. patients*	HLTF		HPP1		hMLH1	
		% Positive	P^{\dagger}	% Positive	P^{\dagger}	% Positive	P^{\dagger}
Size of tumor			0.03		0.021		0.047
T ₁ + T ₂ + T ₃	90	28		11		20	
T ₄	13	46		23		46	
Nodal status			0.514		0.026		0.494
N ₀	54	20		6		24	
N ₁	49	22		20		22	
Distant metastases			0.009		<0.001		0.181
M ₀	86	16		5		26	
M ₁	17	47		53		12	
Localization			0.049		0.156		0.100
Right [†]	25	2		4		12	
Left [‡]	78	20		14		27	
Colon	60	15	0.054	7	0.061	22	0.408
Rectum	43	30		19		26	
Grading			0.872		0.796		0.316
G1	3	33		0		0	
G2	53	21		13		19	
G3	45	22		13		29	
UICC stage			0.042		<0.001		0.648
I	15	20		7		27	
II	34	15		3		24	
III	37	16		5		27	
IV	17	47		53		12	

*For one patient, tumor localization and information on TNM staging was not available.

[†] P values were calculated by the χ^2 test.

[‡]Right colon includes cecum through transverse colon.

[§]Left colon includes descending colon through rectum.

^{||}Relative risk was calculated for G3 versus G1 and G2. G3 includes all cases classified as G3 or G2-G3.

using these cutoff values as well as qualitatively. As results did essentially not differ due to the low cutoff values, only the qualitative results are shown.

Correlation of serum methylation status with clinicopathologic data. The methylation status of *HPP1*, *HLTF*, and *hMLH1* in the pretherapeutic sera of the 104 patients of the test set was analyzed for association with clinicopathologic data. No correlation between methylation status of these genes and age, gender, and histologic grading was found (Table 4; data not shown). Methylation of *HPP1*, *HLTF*, and *hMLH1* significantly correlated with tumor size ($P = 0.03$, 0.021 , and 0.047 , respectively). Only *HPP1* methylation correlated with nodal status ($P = 0.026$). Methylation of *HLTF* and *HPP1* was found significantly more frequent in patients bearing metastases than in those who did not ($P = 0.009$ and $P < 0.001$, respectively, versus $P = 0.181$ for *hMLH1*). Methylation of *HLTF* and *HPP1* was detected more frequently in patients with higher tumor stages than in patients with lower tumor stages ($P = 0.042$ and $P < 0.001$, respectively). As CEA is the only serum marker that has been recommended to be added to the established tumor-node-metastasis (TNM) staging system (30), we sought to correlate CEA levels with the presence of DNA methylation. For CEA analysis, serum was available for 77 patients of the test set. Using the recommended cutoff level for CEA of ≥ 5 ng/mL, we found that positivity for CEA was significantly correlated with the presence of serum methylation of *HPP1* ($P = 0.001$) and the presence of methylation of *HPP1* and/or *HLTF* ($P = 0.001$). In contrast, the correlation of CEA with *HLTF* was not significant ($P = 0.056$) and there was no correlation between CEA and *hMLH1* ($P = 0.571$).

Analysis of prognostic significance of DNA methylation in serum. The association of clinicopathologic data and pretherapeutic serum methylation status of the genes *HPP1*, *HLTF*, and *hMLH1* with clinical outcome was analyzed in 77 patients with known follow-ups. Statistical analysis revealed prognostic significance for tumor size, the presence of distant metastases, and higher UICC tumor stage ($P = 0.025$, 0.034 , and 0.025 , respectively; Table 5). Of the three genes tested, the presence of methylation of *HLTF* or *HPP1* was significantly correlated with a poorer prognosis (Fig. 1; Table 5). The prognosis of patients with methylation of *HPP1* or *HLTF* in serum was associated with a relative risk of death of 5.1 [95% confidence interval (95% CI), 2.2-11.6; $P = 0.001$] and 3.0 (95% CI, 1.4-6.4; $P = 0.008$), respectively. Also in combination, these two genes had prognostic significance. Patients with methylation of *HLTF* and/or *HPP1* had a relative risk of death of 4.2 (95% CI, 2.0-9.0; $P < 0.001$). A subanalysis revealed that the prognosis of stage II patients was also significantly worse when *HPP1* and/or *HLTF* were methylated with a relative risk of death of 11.8 (95% CI, 1.9-74.7; $P = 0.04$).

The multivariate analysis included tumor size, lymph node metastases, distant metastases, age, and methylation of *HPP1* and/or *HLTF*. In the Cox proportional hazards regression analysis of independent variables tumor size ($T_1 + T_2$ versus $T_3 + T_4$), the presence of lymph node metastases, the presence of distant metastases, and age did not attain statistical significance at the α level of 0.05 ($P = 0.735$, 0.62 , 0.735 , and 0.382 , respectively). Serum methylation of *HPP1* and/or *HLTF* DNA was found to provide independent prognostic information associated with a relative risk of death of 3.4 (95% CI, 1.4-8.1; $P = 0.007$; Table 6).

Table 5. Results of univariate analysis

Variable	No. patients who died/total no.	Relative risk of death (95% CI)*	P*
Size of tumor			0.025
$T_1 + T_2 + T_3$	21/66		
T_4	7/10	1.3 (1.1-1.6)	
Nodal status			0.065
N_0	10/39		
N_1	18/37	1.4 (1.0-3.8)	
Distant metastases			0.034
M_0	19/61		
M_1	9/15	2.5 (1.1-5.6)	
Localization			0.873
Right [†]	6/17		
Left [‡]	22/60	1.1 (0.4-2.7)	
Colon	12/40		
Rectum	16/37	1.3 (0.9-1.9)	0.198
Grading			0.363
G_1	0/1		
G_2	13/38		
G_3^{\S}	14/35	1.2 (0.6-2.6)	
UICC stage			0.025
I	3/10		
II	5/24	0.7 (0.2-2.9)	
III	11/27	2.1 (0.7-6.0)	
IV	9/15	1.9 (0.8-4.5)	
HLTF methylation			0.008
Unmethylated	17/60		
Methylated	11/17	3.0 (1.4-6.4)	
HPP1 methylation			0.001
Unmethylated	20/68		
Methylated	8/9	5.1 (2.2-11.6)	
hMLH1 methylation			0.425
Unmethylated	20/59		
Methylated	8/18	1.4 (0.6-3.1)	
HPP1 ± HLTF			<0.001
Unmethylated	14/57		
Methylated	14/20	4.2 (2.0-9.0)	

*95% CIs and P values were calculated by the Cox proportional hazards regression analysis.

[†]Right colon includes cecum through transverse colon.

[‡]Left colon includes descending colon through rectum.

[§] G_3 includes all cases classified as G_3 or as G_2 - G_3 . The relative risk was calculated for G_1 and G_2 versus G_3 .

Discussion

Aberrant hypermethylation of CpG islands is a hallmark of cancer. Its prognostic value has been investigated in primary colorectal cancers. For example, $p16^{INK4a}$ methylation has been found to be associated with shortened survival in two (17, 31) but not in another study (32), and methylation of *ID4* and *MYOD1* has been reported to be correlated with shortened survival. Using different marker panels defining CIMP status, CIMP⁺ was found to correlate with a worse prognosis in one (33) but not in another study (11). These controversial results are most likely due to different marker panels and different methods used for the analysis of DNA methylation. A recently proposed panel of five highly specific methylation markers might prove useful in the future (13). As only few data have been reported on the analysis of methylated DNA in the serum of patients with colorectal cancer, we did a study to identify serum methylation markers providing prognostic information

on patient survival. Due to the lack of a recommended standard marker panel at the start of the study, we chose markers that have been reported to be methylated in colorectal tumors with different frequencies and only rarely methylated in normal colon.

Methylation of the tumor suppressor gene *APC*, of the DNA repair enzyme gene *hMLH1*, and of the cell cycle regulator *p16^{INK4a}* has been reported to occur in 10% to 30% of cases (31, 34–38). *HIC1*, *HLTF*, *HPP1/TPEF*, *RASSF1A* (RAS association domain family protein), and *WT1* have been shown to be methylated in 50% to 80% of colorectal cancers (39–47). Members of the soluble frizzled related proteins family are methylated and thereby transcriptionally silenced in 30% to 90% of colorectal cancers (48, 49). Using a gene

Table 6. Multivariate analysis

Variable	Relative risk of death (95% CI)	P
Size of tumor (T ₁ + T ₂) vs (T ₃ + T ₄)	0.9 (0.5-1.6)	0.735
Lymph node metastases Yes vs no	1.9 (0.9-4.3)	0.620
Distant metastases Yes vs no	1.3 (0.5-3.2)	0.735
Age	1.0 (1.0-1.1)	0.382
HPP1 ± HLTF vs unmethylated	3.4 (1.4-8.1)	0.007

evaluation set of 12 markers, we identified *HPP1*, *HLTF*, and *hMLH1* to be promising methylation markers in the serum of patients with colorectal cancers as they were not methylated in the serum of healthy controls and were more frequently methylated in metastatic than in local disease.

We identified methylation markers in serum that correlated with clinicopathologic data and prognosis. The detection of methylation of *HLTF* or *HPP1* was significantly associated with tumor size, metastatic disease, and tumor stage. *HPP1* methylation was also found more frequently in patients with a positive nodal status. *hMLH1* methylation only correlated with tumor size. Serum methylation of either *HLTF* or *HPP1* or the combination of both was associated with a worse prognosis. Multivariate analysis revealed that serum methylation of *HPP1* and/or *HLTF* was a prognostic factor independent of the classic staging variables tumor size, lymph node, and distant metastases as well as of age. To the best of our knowledge, this is the first study showing that serum DNA methylation is an independent prognostic marker in patients with colorectal cancer. Another recently published study that also analyzed serum DNA methylation in patients with colorectal cancer by use of the markers *HLTF*, *hMLH1*, and *APC* (20) failed to show serum DNA methylation as an independent prognostic factor. This might be due to the markers chosen and the relatively small number of cases analyzed.

The current gold standard for determining prognosis in patients with colorectal cancer is the extent of disease at time of diagnosis as defined by the Dukes' and TNM staging systems (50). Whereas the TNM staging system is highly predictive of outcome at the extremes (e.g., prognosis of stage I versus stage IV tumors), it is less informative for intermediate groups. It would therefore be helpful to identify markers either in the tumor or in the serum of affected patients which reliably allow identifying patients at risk. Thus far, many protein serum markers have been studied, but the only serum marker of prognostic significance suggested to be added to the established staging systems is CEA (30) as it has been shown that preoperative measurement of CEA in serum can provide prognostic information in Dukes' B or equivalent stages (51). We found that positivity for CEA significantly correlated with methylation of *HPP1* in the serum.

One important application of molecular serum markers could be the identification of curatively resected patients at high risk of recurrence who would profit from adjuvant chemotherapy. It has been shown previously that *CIMP*⁺ status in primary colorectal cancers provides predictive information on the survival benefit from 5-fluorouracil chemotherapy in stage III patients (15). Detection of methylated DNA in serum

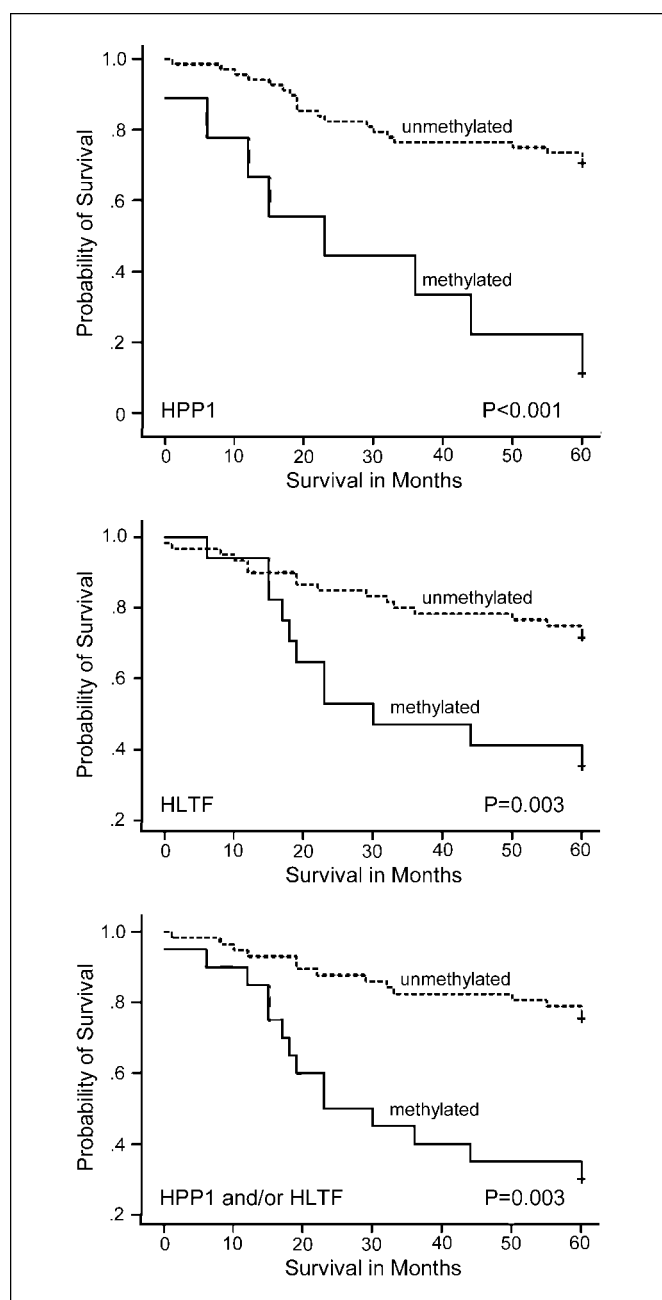


Fig. 1. Kaplan-Meier analysis of the probability of survival.

has also been used to monitor efficacy of adjuvant therapy in breast cancer patients (52). In a subanalysis of stage II patients, we found methylation of HPP1 and/or HMTF to be significantly associated with a worse prognosis. Although our data are limited due to small patient numbers in the subgroup, these provide additional evidence of the potential of serum DNA methylation as a prognostic marker in colorectal cancer.

In conclusion, the present study provides evidence that the detection of methylation of specific genes in the serum of

patients with colorectal cancer is associated with higher mortality. Determination of DNA methylation in serum has the potential to become an independent pretherapeutic predictor of outcome.

Acknowledgments

We thank Dr. M. Zimmermann of Maria-Theresia Clinic in Munich for the help in gathering patient data.

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