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DNA Methylation in Serum and Tumors of Cervical Cancer Patients

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ABSTRACT

Purpose: **Promoter hypermethylation has been recognized to play an important role in carcinogenesis. Numerous studies have demonstrated tumor-specific alterations, such as aberrant promoter hypermethylation, in DNA recovered from plasma or serum of patients with various malignancies. The aim of this study was to investigate the methylation status of various genes in cervical cancer patients and their association with clinicopathological characteristics and outcome of the disease.**

Experimental Design: **The methylation status of *CALCA*, *hTERT*, *MYOD1*, *PGR* (progesterone receptor), and *TIMP3* was investigated in serum samples from 93 cervical cancer patients and 19 corresponding tissue samples using the MethyLight technique.**

Results: **Aberrant promoter hypermethylation was detected in any of these genes in 87% (81 of 93) of the serum samples studied. Methylation of *MYOD1* was detected more frequently in advanced stage. All of the genes found to be methylated in serum samples were also methylated in the corresponding tissue sample, except in one patient. Patients with unmethylated *MYOD1* serum DNA had significantly better disease-free ($P = 0.04$) and overall survival ($P = 0.02$) in comparison with patients with methylated *MYOD1*.**

Conclusions: **To the best of our knowledge, this is, thus far, the largest study investigating aberrant promoter hypermethylation in serum samples from cancer patients and the first study investigating methylation patterns in sera of cervical cancer patients. Our results suggest that serological detection of *MYOD1* promoter hypermethylation may be of potential use as a prognostic marker for discriminating cervical cancer patients at high risk for lymph node metastasis or relapse. Additional studies, including a panel of additional genes, are necessary to elucidate the role of aberrant methylation in serum as a tool for surveillance of cervical cancer.**

INTRODUCTION

Cancer of the uterine cervix is an important cause of death in women worldwide (1). Many studies have investigated clinical and histopathological characteristics as prognostic factors for cervical cancer. Uni- and/or multivariate analysis have revealed that stage, pelvic lymph node metastasis, tumor volume, vascular invasion, and depth of invasion can be prognostic factors for recurrent disease (2, 3). However, new molecular and biochemical approaches for the recognition and treatment of high-risk patients are needed to improve survival and avoid overtreatment of low-risk patients. Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasias (4). It has been increasingly recognized over the past 4–5 years that the CpG islands of a large number of genes that are unmethylated in normal tissue are methylated to various degrees in multiple types of human cancer (4, 5). Aberrant methylation of CpG islands within the promoter regions of several genes such as *p16*, *DAPK* (death-associated protein-kinase), *MGMT* (O^6-methylguanin-DNA-methyltransferase), *E-cadherin* , and *RAR*-β (retinoic acid receptor β) has been identified in cervical cancer (6, 7). Recently, we identified five additional genes, namely *CALCA* (calcitonin-related polypeptide α), *hTERT* (telomerase reverse transcriptase), *MYOD1*, (myoblast determination protein 1), *PGR* (progesterone receptor), and *TIMP3*, as being methylated significantly more frequently in cervical cancer than in normal cervical tissue.[3] The presence of abnormally high DNA concentrations in the serum of patients with various malignant diseases was described years ago (8, 9). The discovery that cell-free DNA can be shed into the bloodstream has generated great interest. Numerous studies have demonstrated tumor-specific alterations in DNA recovered from plasma or serum of patients with various malignancies, a finding that has potential for molecular diagnosis and prognosis. The nucleic acid markers described in plasma and serum include oncogene mutations, microsatellite alterations, gene rearrangements, and epigenetic alterations, such as

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[3] H. M. Müller, A. Widschwendter, G. Goebel, H. Fiegl, E. Müller-Holzner, C. Marth, and M. Widschwendter. A DNA methylation pattern similar to normal tissue is associated with better prognosis in human cervical cancer, submitted for publication.

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Table 1 Methylation of multiple genes in serum samples of cervical cancer patients

Characteristics	n[a]	*CALCA*	*hTERT*	*MYOD1*	*PGR*	*TIMP3*	At least one gene
Stage							
FIGO[b] I	23	57%	0%	4%	74%	4%	78%
FIGO II	24	58%	0%	21%	75%	0%	88%
FIGO III	33	70%	0%	36%	82%	9%	88%
FIGO IV	13	62%	0%	39%[c]	85%	0%	100%
Tumor grade[d]							
1	22	55%	0%	14%	68%	5%	77%
2	50	62%	0%	28%	84%	4%	90%
3	16	81%	0%	38%	81%	6%	100%
Histology							
Squamous	84	63%	0%	27%	77%	5%	87%
Adenocarcinoma	6	67%	0%	0%	83%	0%	83%
Adenosquamous	3	33%	0%	0%	100%	0%	100%
Age							
<50	38	66%	0%	21%	82%	5%	87%
≥50	55	60%	0%	27%	76%	4%	87%
Total	93	62%	0%	25%	79%	4%	87%

[a] *n*, number of cases examined.
[b] FIGO, International Federation of Gynecology and Obstetrics.
[c] $P = 0.029$, χ^2 Pearson.
[d] Tumor grade was unknown in five cases.

aberrant promoter hypermethylation (10, 11). On the basis of these observations, we examined the methylation status of *CALCA*, *hTERT*, *MYOD1*, *PGR*, and *TIMP3* genes in serum samples of cervical cancer patients and compared it with clinicopathological characteristics and outcome of the disease.

MATERIALS AND METHODS

Patients and Samples. A total of 93 patients with invasive cervical cancer (ages 26–96 years; median, 52 years), all treated at the Department of Obstetrics and Gynecology, Innsbruck University Hospital, between 1990 and 1998, were included in this study. Serum samples were taken on the date of diagnosis and before initial treatment. These serum samples were taken from a prior study investigating the presence of serum human papillomavirus DNA in cervical cancer patients (12). Major clinical and histopathological characteristics of patients are given in Table 1. In 19 cases, the corresponding cervical cancer tissue samples were available for analysis. These tissue samples were analyzed in a prior study investigating the methylation status of 25 genes in 65 cervical cancer tissues and 14 normal cervical tissues.[3] All of the patients were followed up after primary treatment at our department at intervals, increasing from 3 months to 1 year, until death or end of the study. The follow-up period ranged from 1 month to 12.4 years (mean, 4.4 years).

DNA Isolation and Methylation Analysis. Genomic DNA from cervical cancer specimen was isolated using the QIAmp tissue kit (Qiagen, Hilden, Germany). Serum samples were treated with SDS and proteinase K at 55°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA. After sodium bisulfite conversion, the methylation analysis was performed by the fluorescence-based, real-time PCR assay MethyLight as described previously (13, 14). Briefly, three sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and two reference sets, β-actin (*ACTB*) and collagen (*COL2A1*), to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using *Sss*I (New England Biolabs)-treated human genomic DNA (heavily methylated). Two separate percentage-of-fully-methylated-reference (PMR) values (separately calculated for *ACTB* and *COL2A1*) were calculated. The percentage of fully methylated molecules at a specific locus was calculated by dividing the *GENE*:*ACTB* ratio of a sample by the *GENE*:*ACTB* ratio of *Sss*I-treated human genomic DNA and multiplying by 100. The abbreviation PMR indicates this measurement. The same calculation was done for the *GENE*:*COL2A1* ratio. The average of both PMR values (calculated for *ACTB* and *COL2A1*) was used as the final PMR. A gene was deemed methylated if the PMR value was >0. To verify the reproducibility of each assay, the normalized value (*Gene*:*ACTB*) of the standard sample was compared between the different PCR runs. The following primers and probes were used for the MethyLight reactions: (*a*) *hTERT:* 5′-GCGTCGGAGGTTAAGGTTGTT-3′ (forward primer), 5′-CTCTCCAAAATTACCGTACGCG-3′ (reverse primer), 5′-6FAM-AACTCGCTCGCCCGCCGAA-BHQ-1-3′ (probe); and (*b*) *PGR:* 5′-TTATAATTCGAGGCG-GTTAGTGTTT-3′ (forward primer), 5′-TCGAACTTCTAC-TAACTCCGTACTACGA-3′ (reverse primer), 5′-6FAM-ATCATCTCCGAAAATCTCAAATCCCAATAATACG-BHQ-1-3′ (probe). The nucleotide sequences of the primers and probes used for the MethyLight reactions for *CALCA*, *MYOD1*, and *TIMP3* were described elsewhere (14).

Statistical Analysis. Associations between categorical variables were tested with Pearson's χ^2 test. Differences in median of PMR values were examined with the Mann-Whitney *U* test. The Kaplan-Meier method was used for univariate survival analysis, and the log-rank test was used to assess the difference between survival curves. Cox's proportional hazards analysis was used to estimate the prognostic effects of various

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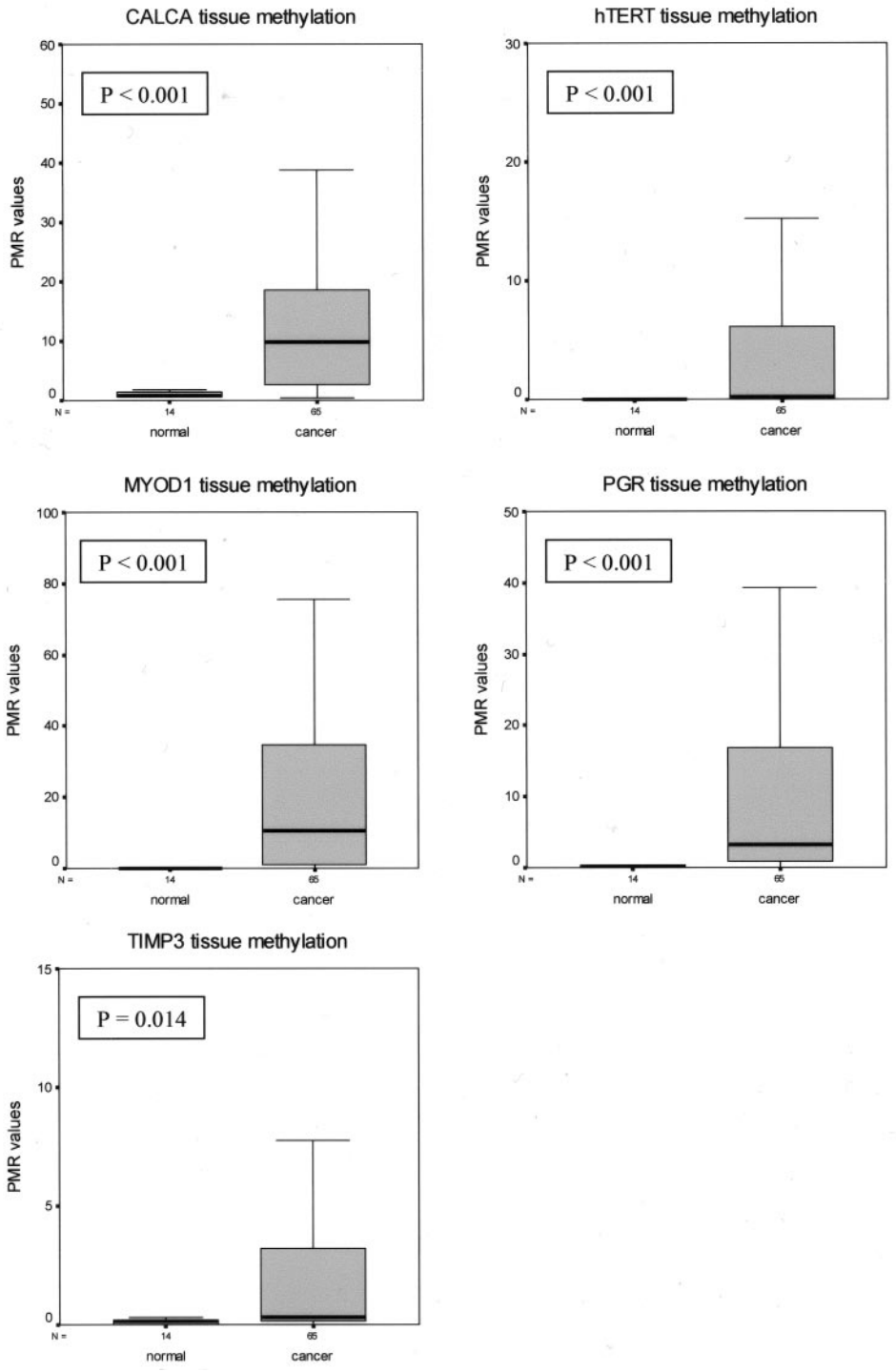

Fig. 1 Methylation [percentage of fully methylated reference (*PMR*) values] in normal and cervical cancer tissues of *CALCA*, *hTERT*, *MYOD1*, *PGR*, and *TIMP3*. Differences in PMR values between normal and cancer tissue were tested with the Mann-Whitney *U* test.

variables. A *P* of less then 0.05 was considered statistically significant. These statistical calculations were performed using SPSS, version 11.0, for Windows.

RESULTS

In a prior study, we investigated the methylation status of 25 genes in 65 cervical cancer tissues and 14 normal cervical tissues.[3] Five genes, namely *CALCA*, *hTERT*, *MYOD1*, *PGR*, and *TIMP3*, were found to be methylated significantly more frequently in cervical cancer than in normal cervical tissue. Comparison of methylation (PMR values) between normal and cancer tissue revealed the most significant results for these five genes (Fig. 1). In the present study, we searched for the presence of promoter hypermethylation of these five genes in serum

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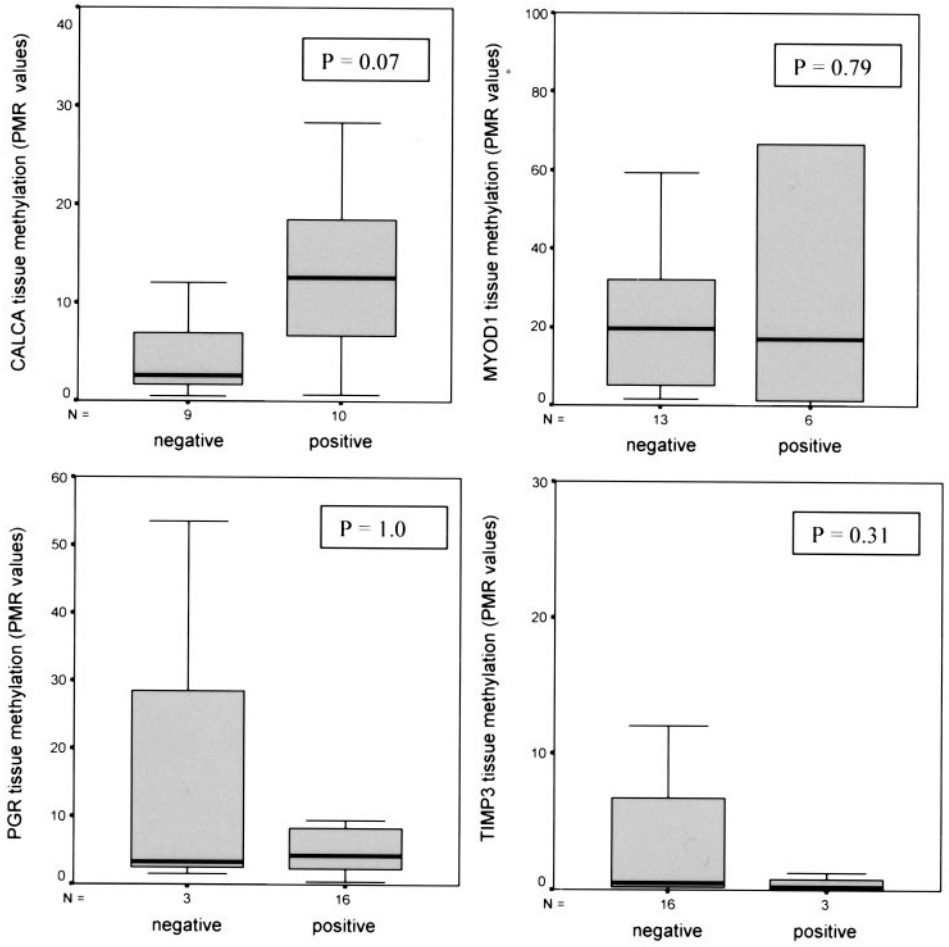

Fig. 2 Comparison of cervical cancer tissue methylation [percentage of fully methylated reference (*PMR*) values] between unmethylated (*negative*) and methylated (*positive*) serum samples for each investigated gene. Box blots for *hTERT* are not shown because no serum sample revealed *hTERT* methylation. Differences were examined with the Mann-Whitney *U* test.

samples of cervical cancer patients taken before initial treatment. Aberrant promoter hypermethylation of any of the genes studied was detected in 87% (81 of 93) of the investigated serum samples. Sixty (74%) of the 81 methylation-positive serum samples showed epigenetic changes in more than one of the genes tested. Promoter hypermethylation of the *PGR* was detected most frequently (79%, 73 of 93) of all investigated genes, especially in serum samples of patients with adenocarcinomas and adenosquamous carcinomas, whereas methylation of *hTERT* was not observed in any of the examined serum samples (Table 1). In advanced stage, methylation of *MYOD1* was detected at a significantly higher frequency than in early-stage cervical cancer ($P < 0.03$). *TIMP3* and *MYOD1* were methylated only in sera of patients with squamous cell carcinoma (Table 1). In all serum samples from patients with a carcinoma classified as tumor grade 3, at least one gene was methylated.

Distant metastases at the date of diagnosis were detected in five patients, and aberrant methylation in serum DNA was observed in all of these cases. Of patients who experienced recurrence with distant metastases ($n = 13$), DNA was methylated in at least one gene in 11 cases, whereas only 2 patients showed no detectable methylation changes in serum DNA. In these 11 cases, DNA methylation was detected in one, two, and three genes in 4, 2, and 5 cases, respectively.

In 19 cases, the corresponding cervical cancer tissue samples were available for analysis. *CALCA*, *MYOD1*, and *PGR* were methylated in all tissue samples, whereas *hTERT* was unmethylated in six cases and *TIMP3* in one case. All of the genes found to be methylated in serum samples were also methylated in the corresponding tissue sample, except one patient who revealed *TIMP3*-methylated serum DNA but no methylation of the corresponding tissue sample. Comparison of cervical cancer tissue methylation (PMR values) between unmethylated and methylated serum samples for each investigated gene revealed no significant results (Fig. 2).

To determine whether the methylation status in serum samples taken at the date of diagnosis has prognostic value, we compared serum DNA methylation of the investigated genes with the clinical outcome of the patients. Fifty-three patients (57%) experienced a recurrence and 51 (55%) died. Median overall survival of all patients was 4.4 years. *CALCA*, *hTERT*, *PGR*, and *TIMP3* methylation status revealed no prognostic significance. Patients with unmethylated *MYOD1* serum DNA had significantly better disease-free and overall survival in comparison with patients with methylated *MYOD1* (Fig. 3). Median survival was 1.9 and 6.1 years for *MYOD1* methylation-positive and -negative patients, respectively. To assess for independent prognostic significance, a Cox proportional hazard model anal-

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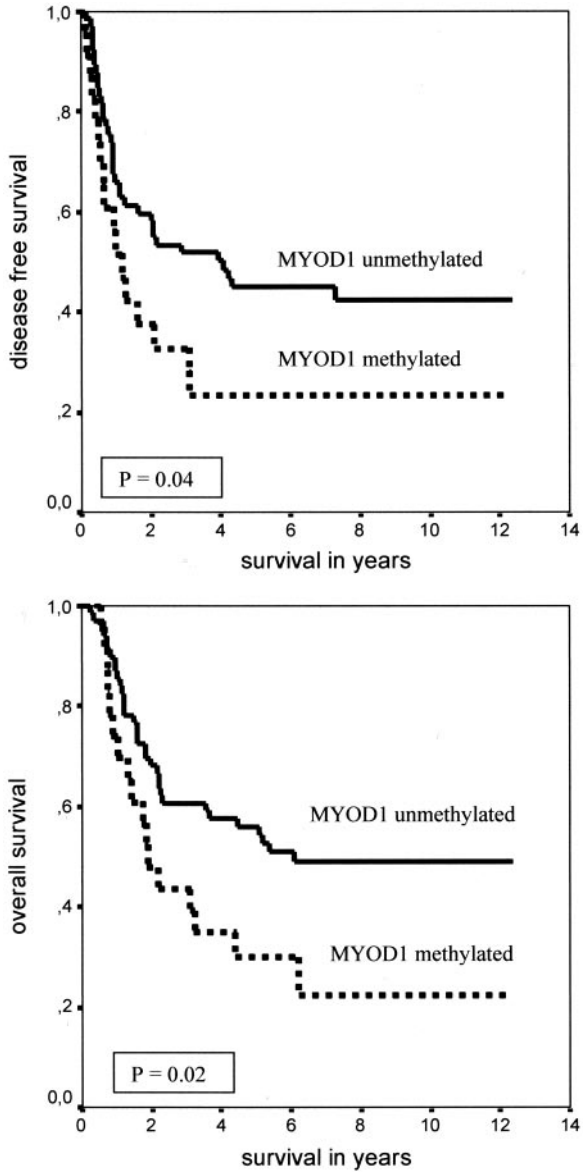

Fig. 3 Disease-free and overall survival according to *MYOD1* methylation status in serum samples.

ysis was performed. The logistic regression model included tumor stage, histology, grade of differentiation, age, and *MYOD1* methylation status. Only International Federation of Gynecology and Obstetrics (FIGO) stage ($P < 0.0001$) was of independent prognostic significance for both disease-free and overall survival.

DISCUSSION

Previous studies have described the importance of DNA methylation in human cancers. Recently, an aberrant methylation pattern was found during the multistage pathogenesis of cervical cancer with an increasing trend to methylation with increasing pathological changes (7). Promoter hypermethylation of various genes is a frequent epigenetic event in cervical carcinoma (6, 7, 15).[3] Epigenetic alterations have been successfully used as indicators of neoplastic serum DNA in patients with various carcinomas (16). To date, no studies have been undertaken to investigate the methylation status of various genes in serum samples of cervical cancer patients. Recently, we identified five genes, namely *CALCA*, *hTERT*, *MYOD1*, *PGR*, and *TIMP3*, as being methylated significantly more frequently in cervical cancer tissue than in normal cervical tissue.[3] In our study, all of the patients with methylated serum DNA revealed the same methylation pattern in the corresponding cervical cancer tissue, except one patient who revealed *TIMP3* methylated serum DNA but no methylation of the corresponding tissue sample. These results are in accordance with previous studies (17–19) and strengthen the evidence that methylated serum DNA is tumor derived. Correlation of PMR values of the investigated genes between tissue and serum showed no significant results. The lack of correlation can be due to several reasons, *e.g.*, different grade of neoangiogenesis, different biological behavior, different development of tumor necrosis, and genetic heterogeneity of the tumors (20). Numerous studies have analyzed the methylation status of cancer-related genes in plasma or serum. The correlation between detection of methylated genes in serum samples and clinical or histopathological features is conflicting in these studies. Whereas several studies report an association with prognosis (19), stage of the disease (21), or occurrence of metastases (18), others found no significant correlation with clinical or histopathological characteristics (22–24).

When investigating methylation patterns of serum DNA and their association with clinical and histopathological parameters, several factors seem to influence the outcome. On the one hand, the choice of appropriate target genes is essential to attain prognostic significance. Although Usadel *et al.* (19) found that methylation of the *APC* (adenomatous polyposis coli) gene in serum samples of lung cancer patients is an independent prognostic factor, Esteller *et al.* (22), analyzing $p16^{INK4a}$, *DAPK*, *GSTP1* (glutathione *S*-transferase), and *MGMT* genes in serum samples of lung cancer patients, observed no correlation between methylation status and prognosis. On the other hand, sample size is a crucial factor to obtain statistically significant results. Wong *et al.* (25) described detection of aberrant *p16* methylation in the plasma and serum of 22 liver cancer patients without observing clinical associations. Incorporating 23 additional patients into the same study, they detected a significant association between the presence of *p16* methylation and the development of tumor recurrence or metastasis (26).

In our study, *MYOD1* and *TIMP3* in serum were methylated only in squamous cell carcinomas, whereas methylation of *PGR* was more frequent in adeno- and adenosquamous carcinomas, implying a specific methylation pattern according to histology. A different pattern of promoter hypermethylation in cervical cancer tissue between squamous cell carcinomas and adenocarcinomas was described previously for *DAPK*, *APC*, and the *HIC-1* (hypermethylated in cancer-1) genes (6). Furthermore, an increase in methylation frequency in serum was observed with a decrease in differentiation of the tumor. In all of the patients with cervical cancer grade 3, at least one of the investigated genes was methylated, whereas only 77% of patients with well-differentiated tumors revealed aberrant methy-

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lation. Additionally, aberrant promoter hypermethylation was detected in all of the patients with distant metastases and in 11 of the 13 patients who experienced recurrence with distant metastasis. These results suggest that multiple methylation is associated with less differentiated and, therefore, more aggressive tumor cells.

Aberrant methylation of *MYOD1* was significantly associated with tumor stage in our study. This is in accordance with a recently published study reporting an association between hypermethylated *APC* DNA in sera of patients with esophageal adenocarcinoma and advanced disease stage (21). Additionally, a higher methylation level of several genes in stage II cervical cancer patients has been described in comparison with patients with a stage I tumor (7). These results suggest that hypermethylation of several genes is associated with advanced-stage and less-differentiated tumor cells and, therefore, that methylation of serum DNA might be a useful marker to identify patients with more aggressive disease.

In a prior study, we analyzed the methylation status of 25 genes in 14 normal cervical tissue specimens and in 65 tissue specimens of cervical cancer patients.[3] Surgically treated lymph node positive patients from this cohort showed statistically significantly higher *MYOD1* PMR values in comparison with lymph node-negative patients.[4] In the present study, patients with unmethylated *MYOD1* serum DNA revealed both better disease-free survival ($P = 0.04$) and better overall survival ($P = 0.02$). These results strengthen the evidence that aberrant *MYOD1* methylation is associated with a more aggressive tumor.

Additionally, an *in vitro* study revealed an increase in the methylation status of *MYOD1* CpG islands during oncogenic transformation (27). Hypermethylation of *MYOD1* has also been described in tissue samples of various malignancies, *e.g.*, breast cancer, colorectal cancers, and malignant lymphoproliferative disorders (28–30). In these studies, aberrant promoter hypermethylation of *MYOD1* was associated with poorly differentiated and more invasive tumors, whereas hypermethylation was not detected in normal tissue

From our findings, we hypothesize that serological detection of *MYOD1* promoter hypermethylation may be of potential use as a prognostic marker for discriminating cervical cancer patients at high risk for lymph node metastasis or relapse, who could benefit from radiochemotherapy, *versus* cervical cancer patients at lower risk for disseminated disease. Additional studies, involving a panel of additional genes, are necessary to elucidate the role of aberrant methylation in serum as a tool for surveillance of cervical cancer.

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[4] See Supplemental Data at http://cancerres.aacrjournals.org.

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