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FAST TRACK

CDH1 AND CDH13 METHYLATION IN SERUM IS AN INDEPENDENT PROGNOSTIC MARKER IN CERVICAL CANCER PATIENTS

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Cervical cancer is the principal cause of death due to cancer in women. Five-year survival rate ranges from 15–80%, depending on the extent of the disease. New predictive markers for relapse may increase survival rates by improving treatment of patients at high risk for relapse. The gene products of *CDH1* and *CDH13*, namely E-cadherin and H-cadherin, play a key role in cell–cell adhesion. Inactivation of the cadherin-mediated cell adhesion system, caused by aberrant methylation, is a common finding in human cancers. To test the hypothesis that *CDH1*/*CDH13* methylation is a prognostic marker in cervical cancer we determined the methylation status of *CDH1*/*CDH13* in serum samples from 93 cervical cancer patients. Methylation analysis was carried out using MethyLight. Aberrant methylation of the 5′-region of *CDH1* or *CDH13* was observed in 43% (40 of 93) of the patients. Cervical cancer patients with unmethylated *CDH1*/*CDH13* in serum samples showed significantly better disease-free survival in univariate and multivariate analysis. Median disease-free survival for *CDH1*/*CDH13* methylation negative and positive patients was 4.3 years and 1.2 years, respectively. Our results suggest that detection of aberrant methylation of *CDH1*/*CDH13* may be of potential use as a marker for selecting cervical cancer patients at high risk for relapse who could benefit from additional systemic therapy.

Key words: *methylation; cervical cancer; E-cadherin; H-cadherin; serum DNA; relapse*

Cancer of the uterine cervix is an important cause of death in women worldwide.[1] Converging evidence from epidemiological and molecular studies suggests that infection of genital human papillomavirus (HPV) is causally linked to the development of cervical cancer.[2] In addition to HPV infection, it is clear that other factors are also involved in cervical carcinogenesis because the majority of patients with HPV-associated lesions do not progress to invasive cancer. Several clinical and histopathological characteristics, namely tumor stage, lymph node metastasis and vascular invasion, have been shown to be prognostic factors for recurrent disease.[3,4] New molecular and biochemical approaches for the recognition and treatment of high risk patients are needed to improve survival and avoid over-treatment of low-risk patients. The gene products of *CDH1* and *CDH13*, namely E-cadherin and H-cadherin, play a key role in cell-cell adhesion. Changes in cell–cell and cell–matrix adhesion accompany the transition from benign tumor to invasive, malignant cancer and the subsequent metastatic dissemination of tumor cells.[5] Decrease or loss of E-cadherin expression is a common finding in many human epithelial cancers including cervical cancer.[5–7] The cadherin-mediated cell adhesion system can be inactivated by several mechanisms. It has been reported that aberrant methylation of CpG islands in the E-cadherin (*CDH1*) as well as in the H-cadherin (*CDH13*) promotor or 5′-region may lead to decreased E-cadherin and H-cadherin expression.[8,9] 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.[10] Based on these observations we investigated the methylation status of *CDH1* and *CDH13* in serum samples of cervical cancer patients for its utility as prognostic marker.

MATERIAL AND METHODS

Patients and samples

A total of 93 patients with invasive cervical cancer (age 26–96 years, median 52 years), all treated at the Department of Obstetrics and Gynecology, Innsbruck University Hospital, between 1990–98 were included in our 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.[11]

Major clinical and histopathological characteristics of patients are given in Table I. Treatment was according to international standards. None of the patients received concurrent chemotherapy and radiotherapy. All patients were followed up after primary treatment at our department, namely at intervals increasing from 3 months to 1 year until death or end of the study. The follow-up period ranged from 1 month–12.4 years (median 3.5 years).

DNA isolation and methylation analysis

Serum samples (300 μl) were treated with SDS and proteinase K (300 μl of 1% SDS, 500 μg/ml proteinase K) at 55°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA. The DNA was re-suspended in 80 μl LoTE buffer (30 mM Tris and 0.3 mM EDTA). Sodium bisulfite conversion of genomic DNA was carried out as described previously.[12] Sodium bisulfite-treated genomic DNA was analyzed by means of the MethyLight, a fluorescence-based, real-time PCR assay, as described previously.[12,13] Briefly, 2 sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and a reference set, β-actin (*ACTB*), to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using *Sss*I-treated (New England Biolabs, Beverly, MA) human white blood cell DNA (heavily methylated). The percentage of fully methylated

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TABLE 1 – METHYLATION OF *CDH1* AND *CDH13* IN SERUM SAMPLES OF CERVICAL CANCER PATIENTS

Characteristics	n[1]	CDH1 (%)	CDH13 (%)
Stage			
FIGO I	23	30	0
FIGO II	24	29	4
FIGO III	33	67[2]	6
FIGO IV	13	23	8
Tumor grade[3]			
1	22	50	0
2	50	34	6
3	16	56	6
Histology			
Squamous	84	42	5
Adeno/adenosquamous	9	44	0
Age			
<50	38	36	5
≥50	55	46	4
Total	93	42	4

[1]Number of cases examined.– [2]$p = 0.005$ χ^2 test.– [3]Tumor grade was unknown in five cases.

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 white blood cell DNA and multiplying by 100. The abbreviation PMR (percentage of fully methylated reference) indicates this measurement. For each MethyLight reaction 10 μl of bisulfite-treated genomic DNA were used. 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 MethyLight reactions: *CDH1*: 5′-AATTTTAGGTTAGAGGGTTATCGCGT-3′ (forward primer), 5′-TCCCCAAAACGAAACTAACGAC-3′ (reverse primer), 5′-FAM-CGCCCACCCGACCTCGCAT-BHQ-1-3′ (probe); *CDH13*: 5′-AATTTCGTTCGTTTTGTGCGT-3′ (forward primer), 5′-CTACCCGTACCGAACGATCC-3′ (reverse primer), 5′-FAM-AACGCAAAACGCGCCCGACA-BHQ-1-3′ (probe).

Statistical analysis

Associations between categorical variables were tested with Pearson's χ^2 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 variables. A p of <0.05 was considered statistically significant. These statistical calculations were carried out using SPSS, version 11.0, for Windows.

RESULTS

Aberrant promotor hypermethylation of *CDH1* and *CDH13* was observed in 42% (39 of 93) and 4% (4 of 93), respectively (Table I). Three of the *CDH13* methylation positive serum samples also showed *CDH1* methylation. We therefore collapsed *CDH1* or *CDH13* methylation for further analysis. *CDH1* methylation was predominantly observed in International Federation of Gynecology and Obstetrics (FIGO) Stage III, whereas distribution of *CDH1* and *CDH13* methylation within the other clinical and histopathological parameters showed no significant differences (Table I).

To determine whether any prognostic significance was attached to differences in *CDH1*or *CDH13* methylation, we compared the clinical outcome of cervical cancer patients with *CDH1*/*CDH13* methylation status. A trend to poorer overall survival for patients with methylated *CDH1*/*CDH13* was observed ($p = 0.09$) (Fig. 1*b*). Cervical cancer patients with unmethylated *CDH1*/*CDH13* in serum samples taken before treatment showed a statistically significant better disease-free survival in comparison to patients with

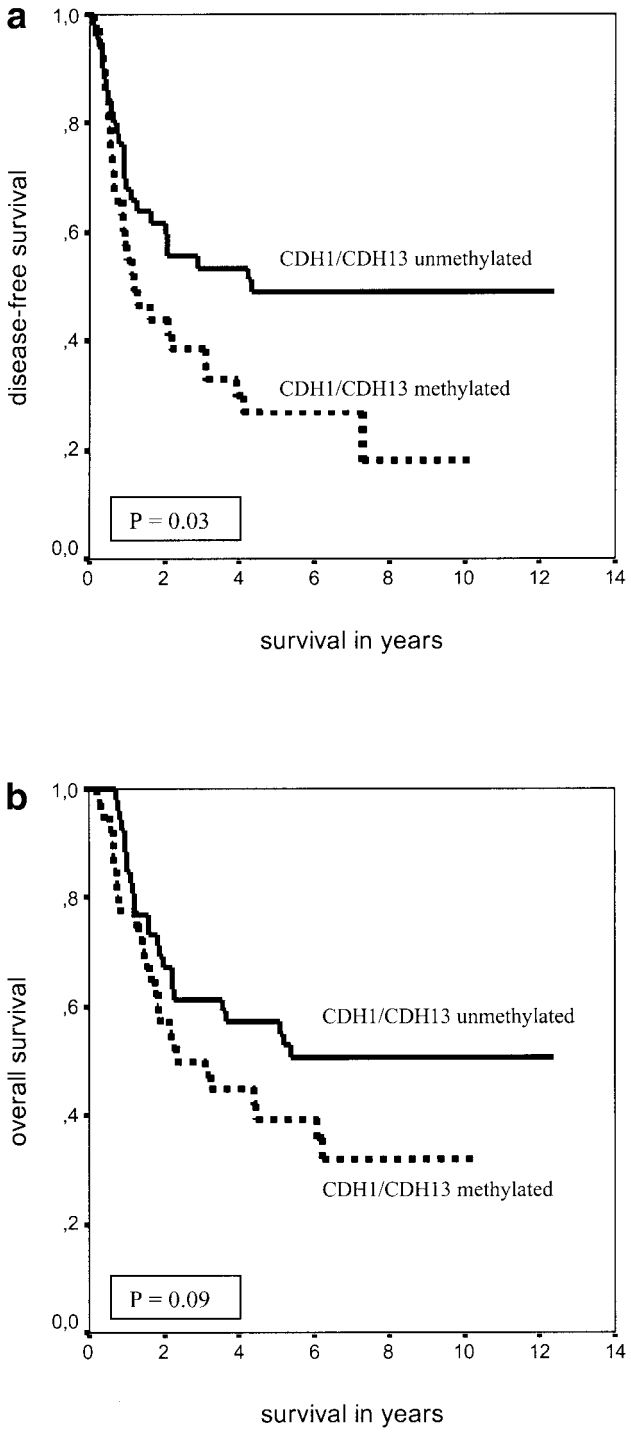

FIGURE 1 – Disease-free (*a*) and overall survival (*b*) according to *CDH1*/*CDH13* methylation status in serum samples.

methylated *CDH1*/*CDH13* ($p = 0.03$) (Fig. 1*a*). Median disease-free survival for *CDH1*/*CDH13* methylation negative and positive patients was 4.3 years and 1.2 years, respectively.

To assess independent prognostic significance a Cox proportional hazard model analysis was carried out including tumor stage, histology, grade of differentiation, age and *CDH1*/*CDH13* methylation status. In addition to tumor stage and age only *CDH1*/*CDH13* methylation status ($p = 0.005$) turned out to be of inde-

TABLE II – MULTIVARIATE ANALYSIS FOR RISK OF RELAPSE

Variable	Relative risk of relapse (95% CI)	*p*
Stage		<0.001
FIGO II (*vs.* FIGO I)	0.6 (0.2–1.6)	0.316
FIGO III (*vs.* FIGO I)	1.3 (0.6–2.9)	0.524
FIGO IV (*vs.* FIGO I)	8.6 (3.3–22.3)	<0.001
Tumor grade		0.132
2 (*vs.* 1)	1.9 (0.9–4.2)	0.092
3 (*vs.* 1)	2.1 (0.9–4.8)	0.065
Histology		
Squamous (vs. adeno/adenosquamous)	0.6 (0.2–1.7)	0.303
Age	0.97 (0.95–0.99)	0.012
CDH1 and/or *CDH13* methylated (*vs.* unmethylated)	2.5 (1.3–4.6)	0.005

TABLE III – MULTIVARIATE ANALYSIS FOR OVERALL SURVIVAL

Variable	Relative risk of death (95% CI)	*p*
Stage		<0.001
FIGO II (*vs.* FIGO I)	0.7 (0.3–1.8)	0.504
FIGO III (*vs.* FIGO I)	1.0 (0.4–2.5)	0.993
FIGO IV (*vs.* FIGO I)	11.1 (4.0–30.4)	<0.001
Tumor grade		0.07
Grade 2 (*vs.* grade 1)	2.3 (1.1–5.0)	0.037
Grade 3 (*vs.* grade 1)	2.5 (1.0–6.1)	0.041
Histology		
Squamous (*vs.* adeno/adenosquamous)	1.3 (0.4–4.3)	0.645
Age	1.0 (0.9–1.0)	0.146
CDH1 and/or *CDH13* methylated (*vs.* unmethylated)	2.5 (1.3–4.8)	0.005

pendent prognostic significance for disease-free and overall survival in cervical cancer patients (Tables II,III). Serum *CDH1*/*CDH13* methylation positive patients had a more than 2-fold risk for relapse and death than did *CDH1*/*CDH13* methylation negative patients.

DISCUSSION

Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasias. It has been increasingly recognized over the past 4–5 years that the CpG islands of a large number of genes, which are unmethylated in normal tissue, are methylated to varying degrees in multiple types of human cancer. Numerous studies have demonstrated tumor-specific alterations like aberrant methylation in DNA recovered from plasma or serum.[10,14] We have demonstrated recently that all genes found to be methylated in serum samples from cervical cancer patients were also methylated in the corresponding tissue samples and are therefore tumor specific.[15]

We investigated the methylation status of *CDH1* and *CDH13* in serum samples of cervical cancer patients. Distribution of *CDH1* and *CDH13* methylation within the clinical and histopathological parameters showed no significant differences except *CDH1* methylation was predominantly observed in advanced FIGO stage. These findings are in accordance with a previously published study investigating *CDH1* methylation in cervical cancer tissue in which only patients with early-stage cervical cancer (FIGO Stages Ib and IIa) were investigated.[16] Therefore, no difference in *CDH1* methylation according to FIGO stage could be observed. Recently, Narayan *et al.*[17] reported a higher methylation frequency of *CDH1* with increasing tumor stage in cervical cancer patients. Additionally, it has been demonstrated that CDH1 is only methylated in cervical cancer tissue but not in normal cervical tissue.[16,17]

Abnormalities of cell adhesion molecule expression like E-cadherin (*CDH1*) and H-cadherin (*CDH13*) occur in various neoplastic diseases, and there is some evidence to suggest that these abnormalities are significant in the progression of certain tumor types including cervical cancer.[7,18] Several mechanisms like tumor hypoxia and necrosis, stimulation of the epidermal growth factor receptor (EGFR) by EGF or TGF-α and mutations of the CDH1 gene have been proposed for cadherin downregulation.[19] Aberrant promotor methylation of *CDH1* and *CDH13* has been described recently to be one of the mechanisms causing loss of or decreased E-cadherin and H-cadherin expression.[8,9] Decreased E-cadherin expression has been shown to be related to enhanced metastasizing activity or more aggressive malignant tumors.[20,21] The higher methylation frequency of CDH1 with increasing tumor stage and the association of *CDH1* and *CDH13* methylation in serum samples with enhanced relapse frequency in our study confirms these results. Nevertheless, patients with loss of cadherin expression caused by other mechanisms may be missed. Until now, it has not been investigated whether one of the above mentioned mechanism alone or a combination of these mechanisms causes loss of cadherin expression. DNA recovered from plasma or serum of patients with various malignancies reflects tumor-specific genetic and epigenetic alterations like methylation of the primary tumor.[10] The simple procedure of blood drawing in combination with a high-throughput analysis like MethyLight opens a feasible approach for a possible routine use of these markers. Inactivation of the cadherin-mediated cell adhesion system, caused by aberrant methylation, is a common finding in human cancers. Therefore, investigation of *CDH1* and *CDH13* methylation as a prognostic parameter in serum samples from patients with various malignancies could be of interest. Our study showed that *CDH1*/*CDH13* methylation is an independent prognostic parameter for both, disease-free and overall survival in cervical cancer patients.

Several studies recently showed a significant reduction in the risk of relapse and death from cervical cancer, which was achieved by concurrent use of chemotherapy and radiotherapy.[22–24] It can be speculated that *CDH1*/*CDH13* methylation positive patients, who are at higher risk for relapse can benefit from radio-chemotherapy. Further studies have to evaluate this predictive value of *CDH1*/*CDH13* methylation.

Our results suggest that detection of aberrant hypermethylation of *CDH1*/*CDH13* may be of potential use as a predictive marker

for discriminating cervical cancer patients at high risk for relapse. Determination of tumor-specific epigenetic alteration in serum or plasma seems to have great potential for molecular diagnosis and prognosis. Additional studies are necessary to elucidate the role of aberrant methylation in serum as a tool for surveillance of cervical cancer.

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