Hypermethylation of promoter regions of the *APC1A* and *p16*^{INK4a} genes in relation to prognosis and tumor characteristics in cervical cancer patients

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Abstract. Hypermethylation of the O^6-*MGMT*, *p14*^{ARF}, *p16*^{INK4a}, *RASSF1A* and *APC1A* genes are unfavourable prognostic markers in colorectal cancer (CRC). We hypothesized that they could be related to prognosis also in cervical cancer. Methylation was studied in DNA extracts from surgical specimens of cancer tissue by novel pyrosequencing methods. In 109 patients (90 squamous cell carcinomas, 19 adenocarcinomas), we found that hypermethylation of the *APC1A* gene promoter occurred in 8.3% of patients, and of *p16*^{INK4a} in 1.8%. *APC1A* hypermethylation was significantly related to more advanced FIGO stage of the tumor (P=0.013), larger tumor diameter (P=0.049) and distant recurrence-free survival (P=0.0007), but not with locoregional recurrence rate, age, HPV status, DNA ploidy, tumor grade or malignancy grading score. We conclude that methylation of the *APC1A* promoter in cervical cancer, as diagnosed by pyrosequencing, is significantly related to major biological characteristics of the tumor, and may be a new predictor of poor prognosis in cervical cancer.

Introduction

Epigenetic regulation through methylation or demethylation of CpG sites in CpG rich areas in promoter regions of cancer related genes plays an important role in the pathogenesis of cancer (1). DNA methylation can be used in an activating as well as a silencing function depending on the particular gene. This process is carried out by methyltransferases (2). DNA methylation is particularly common in tumor suppressor (1) and DNA repair genes. Methylation of the CpG sites in the promoter parts of these genes may inactivate them, causing disturbed cell cycle regulation, cell adhesion, or DNA repair.

We have developed new, quantitative methylation assays for five genes frequently implicated in cancer research, O^6-*MGMT*, *p14*^{ARF}, *p16*^{INK4a}, *RASSF1A* and *APC1A* (3-8). The methylation status of their promoter regions has previously been studied in colorectal carcinoma (CRC) using methylation specific PCR (9,10), a technique which has the limitation of only assaying the methylation status of a few CpG sites (i.e., those interfering with the PCR primer binding) and only gives a qualitative indication if the sites are methylated or not; like all 'allele specific' PCR methods, the judgment of presence or absence of a band on an agarose gel is largely a matter of combination of the number of PCR optimization experiments made, the gel band visualization staining agent used, and individual judgment of gel electrophoresis bands. In our opinion, these drawbacks are a real cause of methodological concern which may have stalled progress in understanding the role of DNA methylation in clinical cancer research.

We set out to develop methods to quantitatively measure the methylated fraction of the promoter CpG sites in the above-mentioned five genes. We wanted the PCR methods to involve unique primer binding sequences, devoid of CpG sites where the primers bind, and consequently that amplicon formation will always take place, independently of the CpG methylation status of the promoter region of the gene. Pyrosequencing® technology gives a quantitative measure, in percent methylation, for each CpG site in the studied sequence, thus allowing also for detection of partially methylated CpG sites. We recently developed methods that quantitate a large number of consecutive CpG sites, including the previously studied as well as additional adjacent ones thus providing a more comprehensive picture of the distribution of DNA methylation throughout the promoter regions of the five cancer suppressor genes, O^6-*MGMT*, *p14*^{ARF}, *p16*^{INK4a}, *RASSF1A* and *APC1A* (11). We applied these novel methods in a study of the possible prognostic impact of methylation of these genes in a clinical material of 109 cervical cancer patients.

Materials and methods

Subjects. A small piece of cervical carcinomas was surgically removed from each subject, and the tumor pieces were fresh

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frozen in -70°C. Pieces of normal skin from the same patient were used as control and treated the same way.

To evaluate the correlation between the methylation pattern of the O^6-*MGMT*, *p14^{ARF}*, *p16^{INK4a}*, *RASSF1A* and *APC1A* genes and the treatment outcome of cervical squamous cell cancer, we first short-listed two groups with 11 subjects in each for a pilot study. The groups were 100% matched for tumor stage and the only difference was that one group had a poor prognosis (had recurrence of cervical cancer after radiotherapy) and the other had a good prognosis (no reoccurrence of cervical cancer after radiotherapy). In these 22 patients, promoter hypermethylation was found only in the *p16^{INK4a}* and the *APC1A* genes (1 and 6 subjects, respectively), whereas the other three genes were found to be unmethylated in all patients. None of the samples was methylated on more than one gene simultaneously. To further study the methylation pattern of the two genes, *p16^{INK4a}* and *APC1A*, that had been implicated by the pilot series, 87 additional cervix carcinoma specimens were studied, of which 19 were adenocarcinomas. The tumor pieces from these patients were removed and treated in the same way as for the pilot study patients. Thus, all 109 subjects were available for the prognostic evaluation of the methylation of these two genes.

The present study conforms to the provisions of the Declaration of Helsinki and was approved by the Regional Ethics Review Board in Uppsala. The participants gave informed consent.

DNA isolation and bisulfite treatment of tissue DNA. A piece (1.5x1.5 mm) from each tissue specimen was macrodissected into small pieces using sterile scalpels. DNA was purified from each sample using a QIAamp DNA Mini kit (Qiagen Inc., Valencia, CA, USA). Approximately 1,000 ng of freshly extracted DNA was used for the bisulfite treatment performed with EZ DNA Methylation kit according to the instructions by the manufacturer (Zymo Research, West Katella, Orange, CA, USA) except that the incubation time was shortened to 10 h. In short, DNA was diluted with M-Dilution buffer and was incubated for 15 min at 37°C. After the incubation, CT conversion reagent was added and the samples were incubated at 50°C for 10 h. The samples were kept on ice for 10 min and M-Binding buffer was added. The samples were centrifuged and washed using centrifugation and M-Wash buffer. The bisulfite treated DNA was eluted in 10 µl M-elution buffer and diluted 4 times with TE buffer (10 mmol/l Tris-HCl, 0.05 mmol/l EDTA, pH 7.5).

PCR and Pyrosequencing®. PCR and Pyrosequencing was performed as previously described (11). Our assays target CpG sites in the promoter regions of the studied genes as defined in previous studies (9,10). Using Pyrosequencing technology, each CpG site could be site specifically quantitated and analyzed as a C/T-polymorphism where a 100% C-reading denotes a fully methylated C in the original gDNA sample whereas a 100% T-reading denotes that the original C was unmethylated in the gDNA of the patient. Intermediate C/T percentages denote partial methylation at the level of the sample (for instance due to an admixture of non-neoplastic cells in the specimens to a variable extent). Representative pyrograms of non-methylated and methylated cervical cancer specimens are shown for the *p16^{INK4a}* gene in Fig. 1 and for the *APC1A* gene in Fig. 2.

Table I. DNA methylation status of the *p16^{INK4}* and *APC1A* gene promoters in 109 cervical cancer specimens.

Gene	No. (%)	Mean ± SD (% C/T)	Range (% C/T$_{min}$-% C/T$_{max}$)
p16^{INK4a}	2 (1.8)	37±27	18-56
APC1A	9 (8.3)	35±12	20-51

Number of tumors that showed methylation, and mean (±SD) and range of methylated fraction.

Statistical analysis. Comparison of means by t-test, and one-way-ANOVA's were calculated by the SPSS software version 15.0. Proportions were analysed by Pearson χ^2-test and p<0.05 was regarded as statistically significant.

Results

Promoter methylation data. Pyrosequencing analysis was used to get a quantitative measure of the methylation status of the promoter regions of five different cancer related genes; O^6-*MGMT*, *p14^{ARF}*, *p16^{INK4a}*, *RASSF1A* and *APC1A*. Only *p16^{INK4a}* and *APC1A* demonstrated any methylation in the pilot study, and we selected these two genes for a more extensive study comprising an additional 87 cancer specimens. One additional patient showed methylation of the promoter region of *p16^{INK4a}* and three additional patients had methylation of the *APC1A* gene. None was methylated in both genes simultaneously. In the complete series of 109 evaluable cervical carcinomas (FIGO stages I-IV), a total of 9 patients (8.3%) showed hypermethylation of the *APC1A* gene. The rate of hypermethylation was similar in patients with squamous cell carcinomas (7/90 cases; 7.8%) and in adenocarcinomas (2/19 cases; 10.5%). A summary of the findings in all 109 cervical cancer samples studied is given in Table I.

The methylation pattern found in the promoter regions of the genes seems to be consistent through the entire promoter sequence of each studied gene within a given patient, i.e. if methylation was detected in a sample all the studied CpG sites in the entire promoter region of that subject were methylated, and not just a few of the CpG sites, as seen in Figs. 1 and 2. The mean methylated fraction given in Table I is the mean value of the CpG sites of the specified gene in all individual methylation-positive patients. No sample reached 100% methylation of its CpG sites. Partial methylation can probably in some cases be attributed to admixture of non-neoplastic cell types present in the tissues to a varying extent.

Relation to clinical outcome. Early carcinomas (FIGO stages I-II) were hypermethylated in 5.4% and more advanced cases (FIGO stages III-IV) in 23.5% (Pearson χ^2-test, P=0.013). Methylated tumors were significantly larger than non-methylated: mean diameter 52 mm vs. 44 mm (t-test, P=0.049). Fig. 3 shows the overall impact of *APC1A* methylation status on distant recurrence-free survival: prognosis was significantly (P=0.0007) different between methylation-positive and methylation-negative patients. Among squamous cell
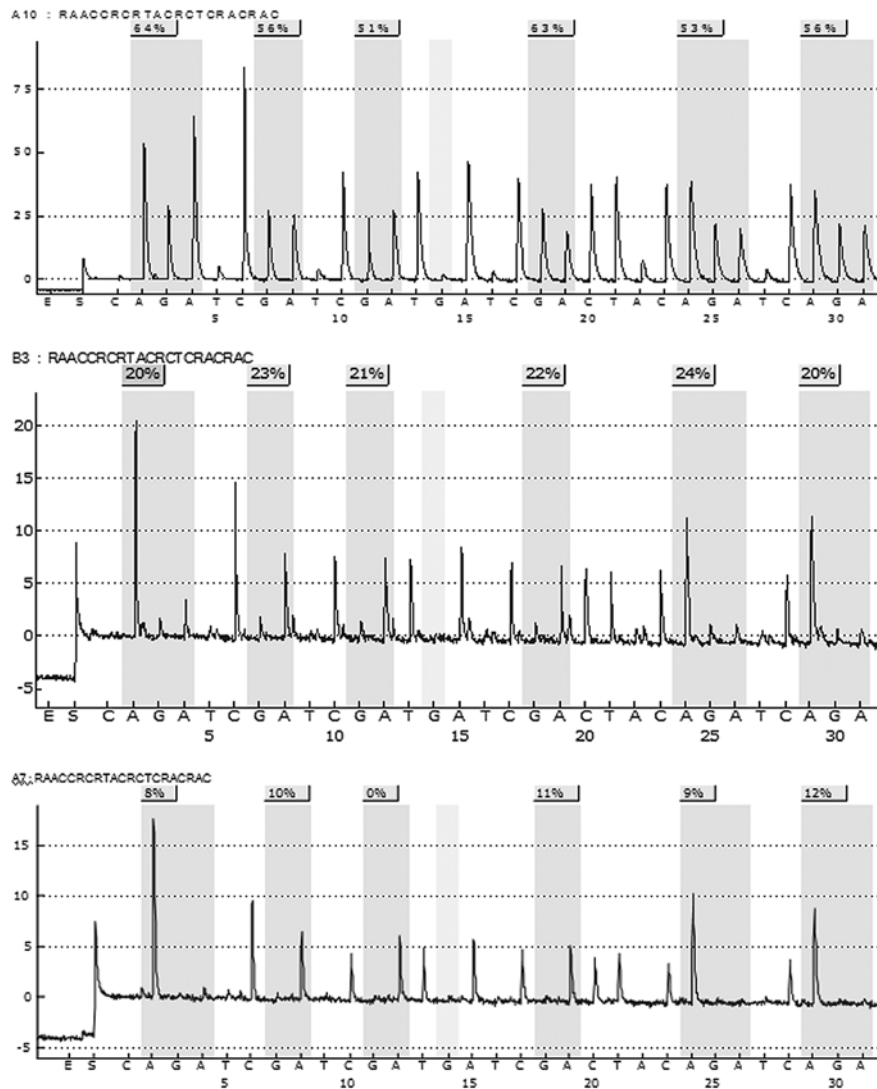
Figure 1. Typical pyrograms of the methylation status of the $p16^{INK4a}$ gene promoter. The pyrograms represent three different patients, showing cervical cancer samples with unmethylated CpG sites (lower panel), sparsely methylated CpG sites (middle panel) and highly methylated CpG sites (upper panel).

carcinomas (Table II) the distant recurrence-free survival was highly significantly associated with the metylation status of the *APC1A* gene: recurrence rate was 9.6% in non-methylated cases and 71.4% in methylated cases (Pearson χ^2-test; P=0.00001). The rate of locoregional recurrences was similar in the two groups. Among adenocarcinomas we did not find such differences between methylated (n=2) and non-methylated (n=17) tumors.

The mean age of the patients with the two types of carcinomas was not significantly different (t-test; P=0.403). Likewise, HPV-status of the tumors, DNA ploidy, tumor grade, and the malignancy grading score (MGS) were not associated with the methylation of the *APC1A* gene. The rate of methylation was not significantly different (Pearson χ^2-test; P=0.549) for tumors with primary cure after radiotherapy and tumors that were not primarily cured.

Discussion

Pyrosequencing offers a unique opportunity to quantitate, site-specifically, the methylated fraction in partially methylated

CpG sites. Several groups worldwide have used and modified Pyrosequencing for methylation assays. It is more efficient and less expensive than combined bisulfite restriction analysis (12) and is more reliable and accurate than a primer extension approach (13). With Pyrosequencing, as many as 10 CpG sites can be studied in the same assay (14,15). CpG sites are thought to be involved in cancer development and have been extensively studied (13,16-22), and it has been shown that exposure of carcinogens affects the DNA methylation patterns (23-25). Furthermore, the significance of the methylation pattern is also shown by the SNRPN gene which is a candidate for diagnostic tests in Prader-Willi syndrome and Angelman syndrome (26). Finally, aberrant methylation patterns in monozygotic twins can cause various disorders (27,28).

The set of cancer suppressor genes studied here was originally devised for applications to colorectal cancer (CRC) (9-11). Two of these genes, $p16^{INK4a}$ and *APC1A* also displayed promoter methylation in 10% of cervical cancer specimens (Table I). We show here for the first time in a clinical setting that promoter methylation of *APC1A* was significantly associated with major biological characteristics of the tumor (Table II), and with
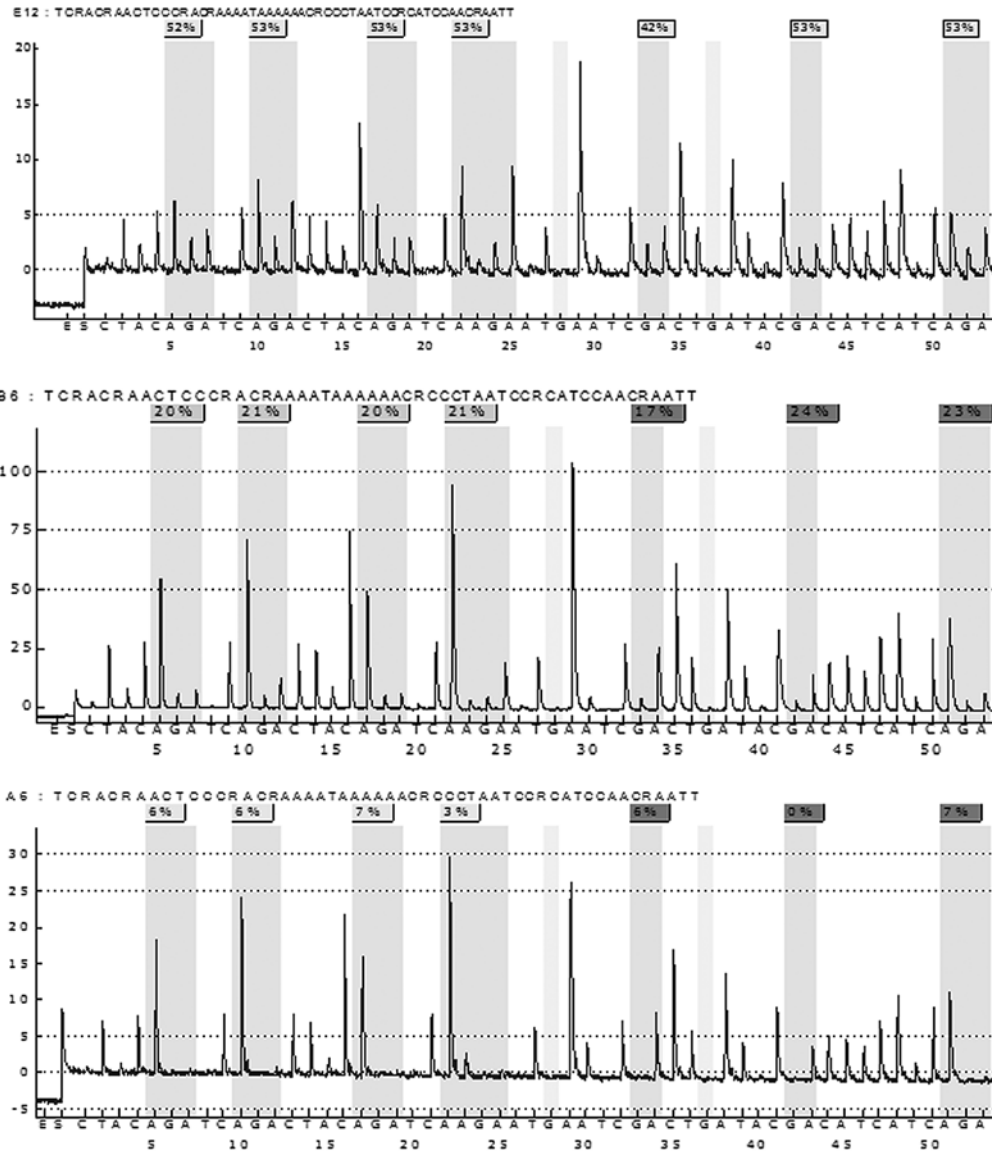
Figure 2. Typical pyrograms of the methylation status of the *APC1A* gene promoter. The pyrograms represent three different patients, showing cervical cancer samples with unmethylated CpG sites (lower panel), sparsely methylated CpG sites (middle panel) and highly methylated CpG sites (upper panel).
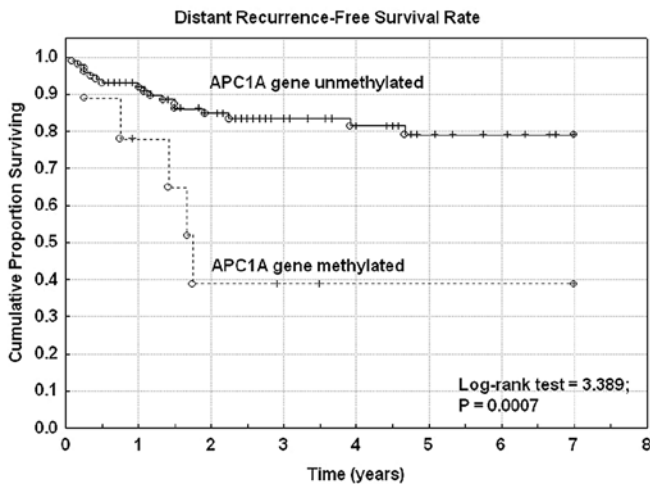

Figure 3. Distant recurrence-free survival rate in 109 cervical cancer patients according to *APC1A* gene promoter methylation status.

distant recurrence-free survival (Fig. 3). In this material, the latter association was mainly driven by squamous cell cancer patients, adenocarcinomas were too few to allow separate assessment of prognosis in relation to methylation status in this subgroup. Increased prevalence of promoter methylation of *APC1A* has also recently been demonstrated in breast cancer tissue (29-31), prostate cancer (32-33), lung cancer (34), ovarian clear cell adenocarcinoma (35), endometrial carcinoma (36), as well as throughout virtually the entire intestinal tract, from oral squamous cell carcinoma (37), to esophagus (38), hepatocellular carcinoma (39), and CRC (40). By contrast, the other four CRC-associated genes studied, O^6-*MGMT*, *p14*^{ARF}, *p16*^{INK4a}, and *RASSF1A* do not seem to be implicated in cervical cancer, at least not by promoter methylation-dependent mechanisms, with the possible exception of *p16*^{INK4a} in a small proportion of cervical cancer patients.

Since methylation of the *APC1A* gene was associated with distant recurrence-free survival one might speculate that the role of the Wnt/β-catenin pathway in tumorigenesis may not

Table II. *APC1A* methylation status according to patient age, tumor size and tumor recurrence rate.

	APC1A unmethylated	*APC1A* methylated	P-value
Age (mean, SD)	65.0 (15.5) years	69.3 (10.6) years	0.472
Tumor size (mean, SD)	44 (11) mm	52 (21) mm	0.049
Tumor stage[a]			
FIGO stage I-II	73	4	
FIGO stage III-IV	10	3	0.026
Primary cure[a]			
Yes (number)	81	7	
No (number)	2	0	0.680
Locoregional relapse[a]			
Yes (number)	10	0	
No (number)	73	7	0.330
Distant relapse[a]			
Yes (number)	8	5	
No (number)	75	2	0.00001

[a]Squamous cell carcinomas (n=90).

be limited to CRC. The relationship we found between *APC1A* methylation status and clinical outcome in cervical cancer is in concordance with its function in cell adhesion and as an inactivator of β-catenin (7,8). This is supported by a recent *in vitro* study showing that demethylation of the APC promoter by hydralazin inhibited growth and increased β-catenin expression of the cervical cancer cell lines HeLa, CaSki and SiHa (41). Mechanistic models are also now being proposed (42,43) to account for the role and importance of *APC1A* in cell cycle regulation and adenoma formation.

In conclusion, we have shown that our novel Pyrosequencing assays of promoters in selected cancer suppressor genes (11) have the potential to be used in clinical diagnostics, and that *APC1A* promoter methylation may be a new marker of unfavourable outcome in cervical cancer patients.

Acknowledgements

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