

Detection of methylation of the promoter region of the MAL and CADM1 genes by pyrosequencing in cervical carcinoma

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Abstract

OBJECTIVE: Cervical cancer is the second most common cancer disease affecting the female population. A key factor in development of the disease is the human papillomavirus infection (HPV). The disease is also impacted by epigenetic changes such as DNA methylation, which causes activation or exclusion of certain genes, and simultaneously the hypermethylation of cytosines in the promoters and turn-off of previously active genes occur. In this study, we focused on the introduction of pyrosequencing for the detection of DNA methylation of the selected CADM1 and MAL genes.

METHODS: DNA was isolated from cytological cervical smear of patients with different types of dysplasia [L-SIL (n=14), ASC-US (n=15), H-SIL (n=1)] and four control samples from healthy women. Prepared samples were further analyzed by bisulfite conversion and subsequent pyrosequencing (Pyromark Q96 ID, Qiagen, Germany). We examined the extent of methylation of CpG islands and as control samples of this method we used a fully methylated and unmethylated DNA. Methylation level (Met level) from each sample was quantified as the mean value [sum of all methylated CpG islands in % / total number of CpG islands (MAL n=4; CADM1 n=3)].

RESULTS: In total, 30 clinical samples and 4 control samples from healthy women were analyzed. By means of the analysis of the CADM1 promoter region, the values of the Met level were obtained [fully methylated DNA (94.83 and 88); completely unmethylated DNA (0 and 0); and control samples from healthy patients (6.825 and 0.825), L-SIL (2.107 and 2.778), ASC-US (7.313 and 3.626), H-SIL (0 and 0)]. By means of the analysis of the MAL promoter region, the values of Met level were obtained [fully methylated DNA (53.25); completely unmethylated DNA (0.875); and control samples from healthy patients (2.925), L-SIL (1.517), ASC-US (2.833), and H-SIL (4)].

CONCLUSION: We introduced a pyrosequencing method for quantification of methylation of CADM1, MAL promoter regions, and detected methylations in clinical samples and also some basal methylation in healthy women.

INTRODUCTION

Cervical cancer is the world's second leading cancer in women and every year about 530,000 cases are diagnosed and about 275,000 women die of this disease (Arbyn *et al.* 2011; Ferlay *et al.* 2008). A major etiological factor in cervical cancer is considered to be the human papillomavirus infection (HPV), of which more than 100 types are currently identified (Sung *et al.* 2013). The development of cervical cancer is a process of several years, involving a precancerous stage known as cervical intraepithelial neoplasia (CIN), which is divided into three stages (CINI-CINIII) (Snijders *et al.* 2006). In addition to histology we also define the cytological finding corresponding to the squamous intraepithelial lesion (SIL). This is divided into a light (L-SIL) and a heavy (H-SIL) form (Solomon *et al.* 2002).

Studies have also shown that infection alone is not sufficient for formation of a carcinoma. For malignant transformation into neoplastic cells, other co-operating factors such as early sexual life at low age, the patient's overall health state, processes suppressing general and local immunity, chronic vaginal infection and high genetic instability, chromosomal aberrations and epigenetic changes in DNA sequence (Sung *et al.* 2013) are also required. In the past, only the malignant transformation of cells was considered to be a consequence of changes in the genome. Nowadays epigenetic changes which affect the incidence and prevalence of disease are also considered to be a similarly binding mechanism.

Epigenetic changes are stable alterations of gene expression without a change in the actual DNA sequence and cause the disease in the absence of a mutation in the gene (Balch *et al.* 2009). These changes include two basic methods of gene turning-off. The first one is the modification of histones and the second one is DNA methylation. Methylation is a covalent chemical modification in which the methyl group is added to the fifth carbon of the cytosine ring (5Mc), thus preventing access of proteins. Most of the DNA methylations run at cytosine-guanine nucleotides, which occur in the human genome in areas called CpG islands.

These islands are present in an unmethylated state and are located at the 5' end (promoter region or the first exon), to 500 bp long and contain at least 55% of CG dinucleotides (Takai *et al.* 2002). The whole process of transmission of the methylated DNA is catalyzed by methyl-transferases (DNMT1, DNMT2, Dnmt3, and DNMT3B) and by a source of -CH₃ (s-adenosyl-methionine), which is converted into s-adenosyl-homocysteine (SAH) (Wajed *et al.* 2001).

In cervical carcinoma, several bio markers have been detected, some of which can be supposed to be of high risk in the development of the disease due to epigenetic changes. These include the *CADM1* gene (cell adhesion molecule 1, *TSLC1*- tumor suppressor in lung cancer 1, *IGSF4*-immunoglobulin superfamily, member 4), which is located at chromosome 11q23.2

and the *MAL* gene (T-lymphocyte maturation associated protein), located in the area 2q11.1. Both genes are tumor suppressor genes which are associated with the progression of cervical cancer (Meijer *et al.* 2013). Bisulfite conversion of these genes could be regarded as an optimal method for detection of CpG methylation, in which hypermethylation in promoters and shutting off of the genes occur (Robertson *et al.* 2005).

MATERIALS AND METHODS

Clinical samples were represented by cervical smears of patients with lesions of the cervix and were collected in cooperation with the Department of Gynecology and Obstetrics, JLF CU, and University Hospital in Martin. All patients were informed about the ongoing studies and signed an informed consent form approved by the Ethical Committee of JLF CU. The cervical smears were taken using LBC medium and then transported to the laboratory. DNA was isolated from cervical smears of patients with different types of dysplasia.

On the basis of the dysplasia's degree of severity, the samples were divided into three groups (Bethesda classification, 2001): 15 samples with a diagnosis of ASC-US (Atypical Squamous Cells of Undetermined Significance), 14 samples with a diagnosis of LSIL (Low-grade Squamous Intraepithelial Lesion) and 1 sample with a diagnosis of HSIL (High-grade Squamous Intraepithelial Lesion). Clinical diagnosis of individual samples was confirmed by histological examination.

DNA was extracted from the cervical cells using a column method with the aid of a DNeasy kit (QIAmp, Qiagen, Germany). We followed the manufacturer's protocol with slight modifications. The isolated DNA was stored at -20 °C for further use. Subsequently the concentration of the sample was determined by measuring the sample purity in a UV spectrophotometer. The quality of the sample, loaded onto 1.5% agarose gel, was determined by means of electrophoresis. In the stored samples with isolated DNA, we subsequently detected hyper-methyl promoters in DNA sequences using methylation-specific PCR with specific primers followed by analyzing DNA samples by pyrosequencing. The samples were modified by bisulfite conversion by means of an EpiTect Bisulfite kit (QIAGEN, USA) according to the attached protocol. For modification, 2 µg of DNA were used in a total amount of 20 µl; the total amount of bisulfite reaction was 140 µl (also containing 85 µl bisulfite mix and 35 µl of DNA Protect Buffer). The DNA Protect Buffer must change from green to blue when added to DNA bisulfite mix. Bisulfite modified DNA was placed into a thermal cycler with the following program: denaturation (95 °C, 5 min), incubation (60 °C, 25 min), denaturation (95 °C, 5 min), incubation (60 °C, 85 min), denaturation (95 °C, 175 min), incubation (60 °C, 25 min), incubation (20 °C). The samples were purified after the last incubation according to the protocol recommended by the

Tab. 1. Amplicon length, number of CpGs and sequence for analysis for genes CADM1 and MAL.

	Amplicon length	Number of CpGs	Sequence to Analyze
CADM1 PyroMark CpG assay (C2)	176 bp	4	TACCGACCGGTTGGCCCCCTAAATCTCCGA
CADM1 PyroMark CpG assay (C1)	173 bp	3	GGCGGCTCTGCACGCCCGGTGCCTCCCTGAAGACACCGA
MAL PyroMark CpG assay	261 bp	4	CTGCGCAGCTAGCGGATCCCGTCCGA

manufacturer. Individual bisulfite reactions were transferred to clean 1.5 ml tubes, to which we added 560 μ l of freshly prepared BL buffer containing RNA Carrier. The test tubes were properly shaken and transferred to the EpiTect Spin column.

The samples were centrifuged at 14,000 rpm for one minute. The overflowed liquid was removed and 500 μ l of buffer BW were added for washing. Centrifugation was repeated and the overflowed liquid removed; then the BD buffer was added and the samples were incubated for 15 minutes at RT. The columns' lids were closed immediately prior to incubation in order to prevent acidification from carbon dioxide in the air. The samples were again centrifuged, the overflowed liquid removed and 500 μ l of buffer BW added for washing. Subsequently the samples were centrifuged and the whole step was once more repeated. After this step the columns' lids were opened and incubated in a thermostat for 5 min at 56°C. At the end the converted samples were eluted in two steps in a volume of 15 μ l and of 10 μ l of EB buffer, through which the final volume of 35 μ l was obtained. The final samples were then stored at -20°C.

After the last elution step, we used the PyroMark PCR kit (Qiagen, USA) for amplification of bisulfite converted DNA. The total volume of PCR reaction was 25 μ l (2 \times PyroMark PCR Master Mix, 10 \times CoralLoad Concentrate, 1 μ l 25 mM MgCl₂, 5 \times Q Solution, 0.24 μ M primer mix, RNase-free water and 20 ng bisulfite treated DNA). Protocol and PCR reaction conditions were as follows: DNA polymerase activation (95°C, 15 min), followed by a three-step cycle of denaturation (94°C, 30 sec), annealing (56°C, 30 sec) and extensions (72°C, 30 sec), a process repeated 45 cycles in a row. Finally, the final extension was carried out (72°C, 10 min). The obtained PCR products were stored in a refrigerator overnight, and then 5 μ l of the product were taken for electrophoresis (1.75% agarose gel, 80 V, 30 min).

The obtained PCR products were analyzed by pyrosequencing using the PyroMark Q96 ID (QIAGEN, USA). We used 10 μ l of the PCR product and subsequently we investigated the extent of methylation in selected CpG islands. We overwrote an area of 261bp for the MAL gene and two areas of 173bp (C1) and 176bp (C2) for the CADM1 gene (Table 1). As control samples, we used completely methylated and unmethylated DNA. For the actual analysis, we followed the information in the protocol provided by the manufacturer.

Tab. 2. Final summary of analyzed samples of promoter regions of MAL and CADM1 genes and obtained values of methylation levels for individual samples.

	Samples (34)	MAL Met level (%)	CADM1 Met level (%)
ASC-US	15	2.833	7.313/3.626
L-SIL	14	1.517	2.107/2.778
H-SIL	1	4	0/0
Healthy women	4	2.925	6.825/0.825
Methylated DNA		53.25	94.83/88
Unmethylated DNA		0.875	0/0

An important step in the pyrosequencing is immobilization of the biotinylated PCR products to the streptavidin-sepharose beads via biotin, which is bound to the 5' end of the primer. In order to immobilize the PCR product to the beads, we prepared a mixture (10 μ l optimized biotinylated PCR product, 1.5 μ l streptavidin-coated sepharose beads, 40 μ l Binding buffer, 28.5 μ l deionized water) and the total volume of a capture was pipetted into each well of the plate. Subsequently the PCR plate was tightly closed with the film, shaken at 1,400 rpm for 10 minutes, then the PCR products were moved using the vacuum pump and washed in the washing device in 70% ethanol, a denaturing solution and wash buffer.

After turning off the vacuum pump, PCR products were released into the plate, which contained 40 μ l of annealing buffer and 0.4 μ M of sequencing primer. Thus prepared, the plate was denatured at 83°C for 2 minutes, then cooled at room temperature and then placed in the device together with the prepared reagent cartridge, in which precise volumes of substrate solution, enzyme solution and individual nucleotides in the chambers were pipetted. The prepared plate and reagent cartridge were then inserted into the device.

RESULTS

We analyzed 30 clinical samples from patients with different degrees of dysplasia (15 samples diagnosed as ASC-US, 14 samples diagnosed with LSIL and one sample with a diagnosis of HSIL) and four control samples from healthy women (Table 2). Samples were collected in collaboration with the Department of

Gynecology and Obstetrics, JFM CU and University Hospital in Martin and diagnosis of individual samples was confirmed by histological examination. Using pyrosequencing we investigated the extent of methylation of individual CpG dinucleotides in the sequences of both genes; with the MAL gene the methylation level for one area was assessed. With the CADM1 gene two areas were assessed.

The methylation level (Met level) from each sample was quantified as the mean of [the sum of all methylated CpG islands (%) / total number of CpG islands (MAL n=4; CADM1 n=3)]. With the analysis of the promoter region CADM1, Met level values were obtained [fully methylated DNA (94.83 and 88), completely unmethylated DNA (0 and 0), control samples from healthy patients (6.825 and 0.825), L-SIL (2.107 and 2.778), ASC-US (7.313 and 3.626), H-SIL (0 and 0)], an exam-

ple of a pyrogram image is shown in Figure 1. With the analysis of the promoter region MAL, Met level values were obtained [fully methylated DNA (53.25), a fully unmethylated DNA (0.875), control samples from healthy patients (2.925), LSIL (1.517), ASC-US (2.833), H thickness (4)], an example of a pyrogram image is shown in Figure 2. As controls, we used the method of fully methylated and unmethylated DNA.

By carrying out bisulfite pyrosequencing of the modified samples from patients with cervical lesions, we found a certain level of methylation in monitored promoter regions, even in samples with a low type of dysplasia. The highest percentage of methylation from the patients with certain types of cervical lesions, was recorded in the sample of the patient confirmed with a high grade intraepithelial lesion (MAL gene, Met level = 4%).

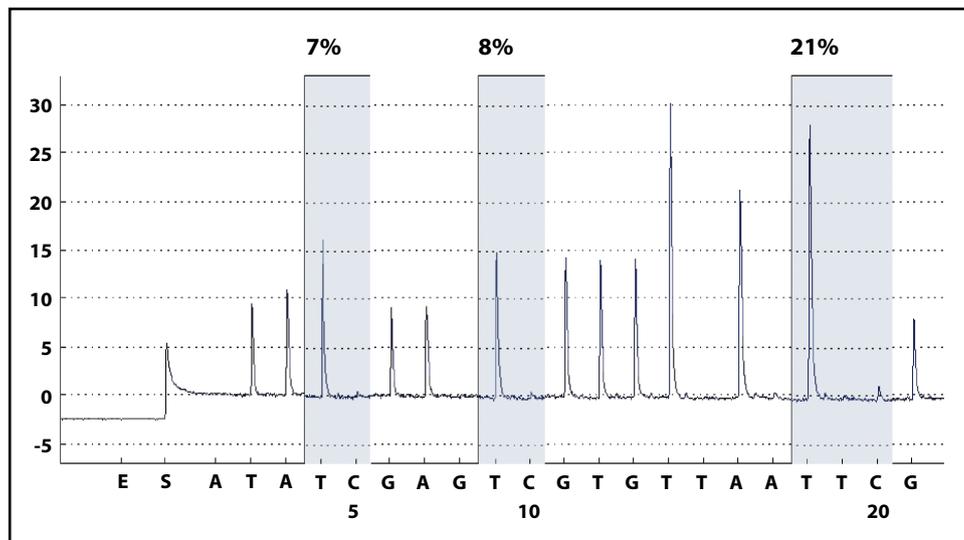


Fig. 1 Result of pyrosequencing of CADM1 promoter region (methylation in monitored CpG areas occurred in 10.11 and 18%) in patients with ASC-US.

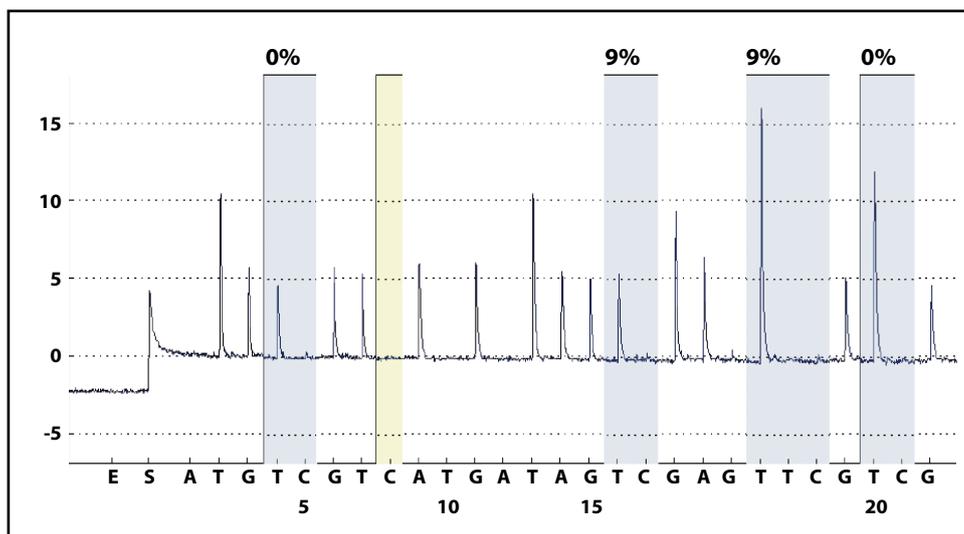


Fig. 2 Result of pyrosequencing of MAL promoter region (methylation in monitored CpG areas occurred in 0.9, 9.0%) in patients with ASC-US.

DISCUSSION

Cervical cancer is the only oncological disease in human medicine in which the etiological factor and sexual transmission by human papillomavirus (HPV) infection are known (Hausen *et al.* 1999). Besides this infection, epigenetic changes also play a significant role in development of carcinoma. These changes are involved in regulation of gene expression of DNA and cause hypermethylation of CpG islands in the promoter region of genes (Portela *et al.* 2010).

In this study we focused on the biomarkers (MAL, CADM1) that could show an association to the factors in cervical cancer. Observing the hypermethylation of the promoter region and hypermethylation of CpG islands of these areas present a perspective method for early detection of cervical lesions that precede the development of carcinoma. We analyzed 30 samples from patients with different types of cervical dysplasia and we examined the methylation

status of promoter regions of the MAL and CADM1 tumor suppressor genes by bisulfite modification of DNA and subsequent pyrosequencing of the PCR products.

Nowadays pyrosequencing is considered to be a gold standard in the detection of hypermethylation because it allows the acquisition of DNA sequences using real-time sequencing during DNA synthesis and quantitative evaluation of methylation of each CpG dinucleotide in the analyzed sequence, and so ensures a more accurate assessment of the significance of the level of methylation of specific genes in the tumor process.

We managed to detect a certain level of methylation in the promoter region of studied samples obtained from patients with a low-type of dysplasia and we also recorded the highest percentage of methylation in a sample from a patient with a high-grade intraepithelial lesion. The study about MAL gene hypermethylation done in the Netherlands did not record a significant incidence of methylation in cervical cancer, though the combination of the CADM1 gene and use of quantitative methylation specific PCR (QMSP) seemed to be a promising biomarker of a tumor process (Overmeer *et al.* 2010; Bierkens *et al.* 2013).

Authors Vasiljevic *et al.* (2014) dealt with methylation of 26 genes, among which CADM1 and MAL genes were analyzed by means of quantitative methylation PCR, but ultimately these genes were not included in their study of pyrosequencing.

In our work, we managed to introduce the method of pyrosequencing to quantify methylation of CADM1, MAL promoter regions; we detected different levels of methylation in clinical samples but also a certain basal methylation in women. Amongst ASC-US and LSIL groups, the resulting degree of methylation was nearly the same for both genes. This indicates that both groups are put into a group with mild dysplasia.

We registered certain irregularities in control samples and in completely unmethylated DNA when the methylation level for the MAL gene was only slightly positive. Such inconsistencies could be avoided by defining new CpG islands in the promoter region, or by analysis of bisulfite dideoxysequencing or of samples in triplicates to pentaplicates .

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