Chapter 2

Sequential gene promoter methylation during HPV-induced cervical carcinogenesis

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Abstract

We aimed to link DNA methylation events occurring in cervical carcinomas to distinct stages of HPV-induced transformation. Methylation Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) analysis of cervical carcinomas revealed promoter methylation of 12 out of 29 tumor suppressor genes analysed, with MGMT being most frequently methylated (92%). Subsequently, consecutive stages of HPV 16/18 transfected keratinocytes (n=11), ranging from pre-immortal to anchorage independent phenotypes, were analysed by MS-MLPA. Whereas no methylation was evident in pre-immortal cells, progression to anchorage independence was associated with an accumulation of frequent methylation events involving 5 genes, all of which were also methylated in cervical carcinomas. TP73 and ESR1 methylation became manifest in early immortal cells followed by RARβ and DAPK1 methylation in late immortal passages. Complementary methylation of MGMT was related to anchorage independence. Analysis of 9 cervical cancer cell lines, representing the tumorigenic phenotype, revealed in addition to these 5 genes frequent methylation of CADM1, CDH13 and CHFR.

In conclusion, 8 recurrent methylation events in cervical carcinomas could be assigned to different stages of HPV-induced transformation. Hence, our *in vitro* model system provides a valuable tool to further functionally address the epigenetic alterations that are common in cervical carcinomas.

Introduction

Worldwide carcinoma of the uterine cervix is a leading cause of cancer-related death for women (1). The majority of cervical carcinomas are squamous cell carcinomas (SCCs), whereas adenocarcinomas (AdCAs) account for the remaining 15-20% of cases (2, 3).

Development of cervical cancer is causally related to infection with high-risk human papillomaviruses (hrHPVs), predominantly types 16 and 18 (4-6). Although hrHPV can be detected in virtually all cervical SCCs and AdCAs (7-10), progression of a hrHPV positive premalignant lesion to invasive cancer is a rare event. Consistent with the multistep nature of human carcinogenesis, additive host cell alterations drive progression to invasive cancer (11). Insight in these additional events may provide novel biomarkers for risk assessment of hrHPV-infected women. These events may involve chromosomal alterations affecting structure and expression of (candidate) oncogenes and tumor suppressor genes, as well as epigenetic alterations. The latter, including both histone modifications and DNA methylation, provide one mode of tumor suppressor gene silencing (12). DNA methylation generally refers to the addition of a methyl group to the 5' position of a cytosine base preceding a guanine. Methylation of CpG rich sequences, so-called CpG islands, which are often present in gene promoter sequences, usually inhibits gene transcription.

Molecular markers based on DNA methylation, i.e. methylation markers, are of particular interest as recent studies indicate that DNA methylation can be easily detected in cervical scrapes using sensitive PCR based methods like methylation specific PCR (MSP). Moreover, positive MSP results in cervical scrapes represented methylation of respective genes in the underlying epithelium (13, 14) .

To date a number of studies have described aberrant methylation of established or candidate tumor suppressor genes in cervical carcinoma tissues, which include genes involved in apoptosis, WNT signalling, Ras-signalling and tumor invasion and metastasis [reviewed by (15). In several of these studies methylation of up to 16 gene promoters has been tested on cervical cancer biopsies, showing that up to 93% of them were positive for at least one of the methylation markers tested (16-25). However, for most of the genes studied it is still unclear to what extent and at what stage promoter methylation reflects a functionally important step in the transformation process. This information will greatly contribute to the design of a clinically optimal marker panel for high-

grade premalignant cervical lesions and cervical cancer in terms of both sensitivity and specificity.

In order to decipher functionally important steps in HPV-mediated transformation a longitudinal *in vitro* model system of hrHPV-transfected keratinocytes (26) can, next to cervical cancer cell lines, be of great value. We have previously transfected primary human keratinocytes with full-length HPV types 16 or 18, resulting in four immortal keratinocyte cell lines, i.e. FK16A and FK16B containing HPV16, and FK18A and FK18B containing HPV18 (27). With increasing passage these cell lines revealed increasing severity of dysplastic features in organotypic cultures that are reminiscent of the various stages of pre-malignant cervical lesions [Steenbergen et al., 1998b]. Moreover, consecutive passages showed accumulation of chromosomal alterations overlapping with those found in cervical (pre)malignant lesions (28-31) and shared altered expression of certain genes with these (pre)cancer stages (32-34). It is currently, however, unknown to what extent this system also provides a valuable model for studying epigenetic alterations that are common in cervical carcinomas.

A novel method to assess the methylation status of multiple genes is Methylation Specific Multiplex Ligation-dependent Probe amplification (MS-MLPA). MS-MLPA is a PCR-based technique allowing the semi-quantitative detection of changes in DNA promoter methylation of multiple genes in a single reaction (35). Discrimination between methylated and unmethylated targets is based on the annealing of probes containing a recognition site for the methylation sensitive restriction enzyme *HhaI.*

In this study we first assessed by MS-MLPA which genes out of 29 (candidate) tumor suppressor genes are methylated in cervical SCCs and AdCAs. Next, we determined at what stage during HPV-mediated transformation methylation of identified targets became manifest.

Materials and methods

Tissue specimens, cell lines and DNA isolation

Frozen specimens of 16 SCCs and 8 AdCAs were collected during the course of routine clinical practice at the VU University Medical Center in Amsterdam. Serial cryo-sections were made, the outer of which were used for histomorphological assessment. All specimens included contained >70% tumor cells. The inner sections were used for DNA extraction and HPV typing as

described previously (36). This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center.
Two primary keratinocyte cell lineages (EK05-1 and EK94-2) were cultured using conditions described before (37). The cell lines FK16A, FK16B, FK18A and FK18B have been established previously by transfection of primary foreskin keratinocytes with either full length HPV16 (FK16A and FK16B) or HPV18 (FK18A and FK18B) (38). The cells were grown in serum-free keratinocyte growth medium (Life Technologies, Breda, The Netherlands) supplemented with bovine pituitary extract (50µg/ml), epidermal growth factor (5ng/ml), penicillin (100U/ml), streptomycin (100µg/ml) and L-glutamin (2nM). Cells were harvested using 10mM trypsin (Life Technologies).
Anchorage independent cell clones of FK16A and FK18B were obtained by culturing late immortal cells in soft agarose, as described previously (39), resulting in the outgrowth of a limited number of colonies. Of each cell line a single colony was isolated, referred to as FK16ASA and FK18BSA and expanded further. Cervical cancer cell lines SiHa, HeLa and CaSki were obtained from the American Type Tissue Culture Collection (ATCC). The 6 low passage cervical cancer cell lines 778, 808, 866, 879 and 915 were kindly provided by Prof. dr. P. L. Stern and cultured as described previously (40).
From cultured cells DNA was isolated using the QIAmp tissue kit (Qiagen, Hilden, Germany).

Methylation Specific-Multiplex Ligation dependent Probe Amplification

MS-MLPA was performed as published previously (35), using two probe mixtures (P041A and P041B) provided by MRC-Holland BV. In total the probe mixtures contained a panel of probes specific for 29 candidate tumor suppressor genes (Table 1). Some of the genes (i.e. APC, ATM, BRCA2, CHFR, CDH13, CDKN1B, CDKN2A, CDKN2B, ESR1, FHIT, GSTP1, HIC1, MLH1, PTEN, RARβ, RASSF1, STK11, TP73, CADM1 (previously referred to as TSLC1), VHL) were represented by two or three probes that each recognized a different *Hha1* restriction site in the promoter region of the respective genes. In addition, each probe mix contained control probes that lack the *Hha1* restriction site and were used for quantification purposes.
The MS-MLPA procedure is summarized in Figure 1. For each sample 100 ng input DNA was used. After 10 minutes denaturation at 98°C, SALSA MLPA buffer and MS-MLPA probe mix P041A or P041B was added to the DNA, and the mixture was incubated for 1 minute at 95°C, followed by hybridization for

16 hours at 60°C. After hybridization the samples were diluted at room temperature with H_20 and 3 µl ligase buffer A to a final volume of 20µl and divided over two series of tubes. Samples were heated to 49°C, after which a volume of 10 µl of a mix containing 0.25 µl ligase, 1.5 µl ligase buffer B and 5U *Hha1* (Promega, Leiden, The Netherlands) was added. To the second series of tubes an identical mix was added in which Hha1 was replaced by 50% glycerol. Ligation and digestion were performed simultaneously for 45 minutes at 49°C, which was followed by a 5 minutes incubation at 98°C to inactivate the enzymes.

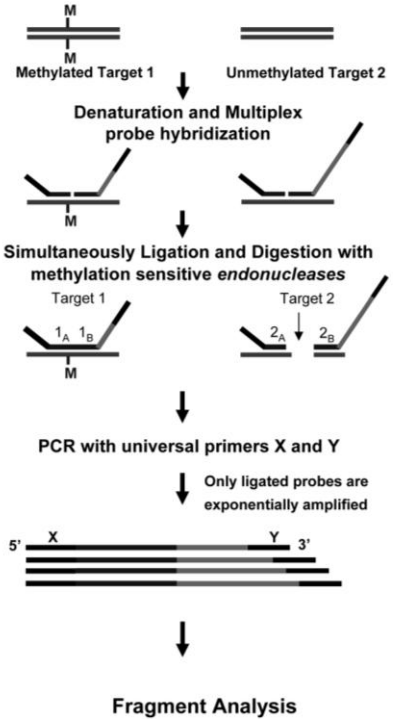


Figure 1. Overview of the MS-MLPA procedure. The gene specific probes spanning a recognition site for the restriction enzyme *Hha1* are hybridized to the target DNA and subsequently ligated and digested with the methylation sensitive enzyme *Hha1*. Undigested probes, i.e. probes of which the recognition sequence is methylated will be amplified. If the CpG site is unmethylated the DNA/probe complex will be digested and no amplification will take place. For each DNA sample the MS-MLPA was performed with and without *Hha1* digestion.

To amplify the ligation products, 5 µl was added to a PCR mix containing 10x SALSA PCR buffer, 1U SALSA polymerase and SALSA FAM PCR primer-dNTP mix (all provided by MRC Holland, Amsterdam, The Netherlands) and

the following PCR protocol was used: 1 minute at 95°C; 35 cycles (30 seconds at 95°C, 30 seconds at 60°C, 1 minute at 72°C); 20 minutes at 72°C.
For fragment analysis 8.75µl Formamide (Sigma-Aldrich, Zwijndrecht, The Netherlands) 0.25µl Gene Scan-500 ROX Size Standard (PE Applied Biosystems, Foster City, California, USA) and 1 µl PCR product was run on an ABI PRISM 3100 Avant (ABI PRISM 3100- Avant Genetic Analyzer by PE Applied Biosystems). Analysis was performed using ABI 3100 Gene Scan 3.7 software. Each amplified fragment was normalized by dividing the area under the peak by the mean of the two flanking control fragments. The percentage of methylation was calculated by dividing normalized peaks in the *Hha1* digested reaction by the normalized peaks in the undigested control reaction. Methylation below the threshold level of 10% was considered background (35). All samples were analysed twice and scored positive for methylation when in both experiments the percentage of methylation for each individual probe was above the threshold. In case of multiple probes for a single gene promoter, the gene was scored positive when ≥1 probe showed methylation.

Table 1. Genes represented by the probe mixtures (41A and 41B). Probes present in both probe mixtures representing the same gene recognise a different restriction site in corresponding promoter regions

41A		41B	
TIMP3	tissue inhibitor of metalloproteinases-3	BRCA1	breast cancer 1
APC	adenomatosis polyposis coli	BRCA2	breast cancer 2
CDKN2A	cyclin-dependent kinase inhibitor 2A p16	ATM	ataxia telangiectasia mutated
MLH1	mutL homolog 1	TP53	tumor protein 53
ATM	ataxia telangiectasia mutated	PTEN	phosphatase and tensin homolog deleted on chr ten
RARB	retinoic acid receptor, beta	SMARCA3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin
CDKN2B	cyclin-dependent kinase inhibitor 2B p15	CHFR	checkpoint with forkhead and ring finger domains
HIC1	hypermethylated in cancer 1	CDH13	cadherin 13
CHFR	checkpoint with forkhead and ring finger domains	TP73	tumor protein p73
CASP8	caspase 8	STK11	serine/threonine kinase 11
CDKN1B	cyclin-dependent kinase inhibitor 1B p27	VHL	von Hippel-Lindau
PTEN	phosphatase and tensin homolog deleted on chr ten	GSTP1	glutathione S-transferase pi
BRCA2	breast cancer 2	HIC1	hypermethylated in cancer 1
CD44	CD44 molecule	ESR1	estrogen receptor 1
RASSF1	RAS association domain family 1A	RB1	retinoblastoma 1
DAPK1	death associated protein kinase 1	FHIT	fragile histidine triad gene
VHL	von Hippel-Lindau	STK11	serine/threonine kinase 11
ESR1	estrogen receptor 1	CADM1	cell adhesion molecule 1
RASSF1	RAS association domain family 1A	MGMT	O6-methylguanine-DNA methyltransferase
TP73	tumor protein p73	CDKN1B	cyclin-dependent kinase inhibitor 1B p27
FHIT	fragile histidine triad gene	APC	adenomatosis polyposis coli
CADM1	cell adhesion molecule 1	CDKN2B	cyclin-dependent kinase inhibitor 2B p15
CDH13	cadherin 13	CDKN2A	cyclin-dependent kinase inhibitor 2A p16
GSTP1	glutathione S-transferase pi	RASSF1	RAS association domain family 1A
MLH1	mutL homolog 1	RARB	retinoic acid receptor, beta

Methylation-Sensitive Single-Nucleotide Primer Extension (Ms-SNuPE)

Primers used to generate the specific MGMT PCR product for Ms-SNuPE analysis were 5'- GTATTAGGAGGGGAGAGATT -3' and 5'- TCTATACCTTAATTTACCAAATAACCC -'3. The PCR was performed in a total volume of 25 µl containing 25ng bisulfite modified DNA. PCR conditions were 8 minutes at 95°C, 35 cycles (30 seconds at 95°C, 30 seconds at 54°C, 45

seconds at 72°C); 4 minutes at 72°C. PCR products were separated on 1% agarose gels and isolated using the GeneClean III kit (Qbiogene, Irvine, USA).
Ms-SNuPE reactions were performed mainly as described before with some modifications (41). Conditions for primer extension reactions were; at 95°C for 1 min, 48°C for 1 min and 72°C for 1 min. The primers used for the Ms-SNuPE analysis were 5'-GGGATTTTTATTAAGCGGG-3' and 5'-GGGATTTTTATTAAGTGGG-3'.
The reaction was performed in a total volume of 10 µl, containing 4 µl of purified PCR product and 6 µl PCR mix consisting of; 10x PCR buffer (MRC-Holland), 10pmol of each Ms-SNuPE primer, 1µCi of either [^{32}P]dCTP or [^{32}P]dTTP and 1U of Taq polymerase (MRC-Holland).
Stop solution was added and samples were denatured for 4 min at 95°C. Of the sample 1.5 µl was loaded on a 15% polyacrylamide gel (7M urea).
Radioactivity was quantitated using a phosphoimager. The percentage of methylation is equivalent to the value of C/(C+T). The specimens were rated positive when methylation level reached 5%.

Statistical Analysis

Methylation percentages for individual genes in SCCs and AdCAs were compared using chi-squared statistical testing. A two-sided p value of ≤ 0.05 was considered significant.

Results

Promoter methylation profiles in cervical SCCs and AdCAs

To firstly identify epigenetic alterations associated with cervical cancer we analyzed promoter methylation of 29 (candidate) tumor suppressor genes by MS-MLPA in 16 SCCs and 8 AdCAs. All carcinomas contained DNA of high-risk HPV types (HPV16 in 10 SCCs and 4 AdCAs, HPV 18 in 1 SCC and 4 AdCAs, HPV33, 35, and 39 each in 1 SCC) or not yet classified HPV types (HPV 67 and 69, each in 1 SCC). An overview of all genes that were methylated in the individual SCCs and AdCAs is shown in Figure 2. In addition, a summary of the overall frequencies of methylation found in SCCs and AdCAs is shown in Table 2. Twelve of the genes showed a positive MS-MLPA result in one or more of the carcinomas tested. SCCs revealed frequent promoter methylation, i.e. in >40% of cases, of CDH13 (9/16: 56.3%), DAPK1 (9/16: 56.3%), MGMT (15/16: 93.8%) and CADM1 (9/16: 56.3%). In AdCAs

frequent promoter methylation of APC (4/8: 50%), CDH13 (7/8: 87.5%), CHFR (4/8: 50%), MGMT (7/8: 87.5%), TIMP3 (5/8:62.5%) and TP73 (7/8: 87.5%) was detected.

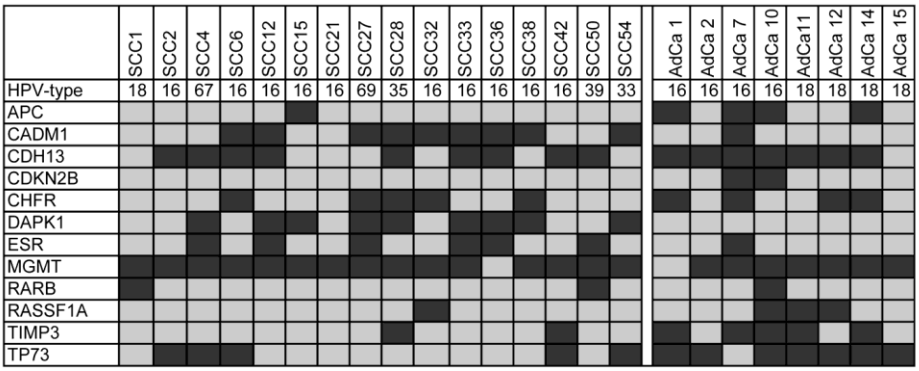


Figure 2. Summary of MS-MLPA results on cervical SCCs (n=16) and AdCA (n=8). Black boxes indicate the presence of promoter methylation; grey boxes represent unmethylated CpGs. For each SCC and AdCA the HPV type present is also shown.

Table 2. Methylation frequencies in SCCs and AdCAs. Genes indicated in *italics* are significantly more frequently methylated in AdCAs than SCCs, and those in **bold** show a significantly higher frequency of methylation in SCCs.

Gene	SCC	AdCa	p value
APC	6.3% (1/16)	50% (4/8)	0.01
CADM1	56.3% (9/16)	6.25% (1/8)	0.04
CDH13	56.3% (9/16)	87.5% (7/8)	0.13
CDKN2B	0%	25% (2/8)	0.04
CHFR	31.3% (5/16)	50% (4/8)	0.37
DAPK1	56.3% (9/16)	0%	0.01
ESR1	37.5% (6/16)	12.5% (1/8)	0.20
MGMT	93.8% (15/16)	87.5% (7/8)	0.60
RARβ	12.5% (2/16)	12.5% (1/8)	1.00
RASSF1A	6.3% (1/16)	37.5% (3/8)	0.05
TIMP3	12.5% (2/16)	62.5% (5/8)	0.01
TP73	31.3% (5/16)	87.5% (7/8)	0.01

MGMT was most frequently methylated in both tumor histotypes, i.e. in 92% of all carcinomas. On the other hand methylation of DAPK1 (p=0.007) and CADM1 (p=0.04) was significantly higher in SCCs, whereas APC (p=0.01), CDKN2B (p=0.04), RASSF1A (p=0.05), TIMP3 (p=0.01) and TP73 (p=0.02) methylation was more common in AdCAs (Table 2). No statistically significant

different methylation profiles were found between HPV 16 positive carcinomas and carcinomas containing other hrHPV types.

Accumulation of methylation events during HPV mediated transformation *in vitro*

To determine at which stage during HPV-mediated transformation methylation of the various genes becomes manifest, we performed MS-MLPA analysis on an *in vitro* model system of HPV transformed keratinocytes and cervical cancer cell lines. In previous studies, we and others have shown that at least 4 phenotypes can be distinguished during HPV-mediated transformation; (1) extended but still finite lifespan (pre-immortal), (2) immortalization (3) anchorage independent growth and (4) tumorigenicity (42-45). We analyzed 2 isolates of primary keratinocytes as normal controls and one HPV18 transfected cell culture (FK18B) representing the pre-immortal stage. Moreover, early passages (passage 39-61) and late passages (passage 75-124) of 2 HPV16 and 2 HPV18 immortalized cell lines (FK16A, FK16B, FK18A and FK18B) were included, as well as anchorage independent clones of HPV16 (FK16ASA) and HPV18 (FK18BSA) containing cell lines. Finally, 9 hrHPV positive cervical cancer cell lines (SiHa, HeLa, CaSki, 778, 808, 866, 873, 879 and 915), of which the latter 6 were tested at low passage, were included as representatives of the tumorigenic stage. A schematic representation of the cell lines analyzed is shown in Figure 3.

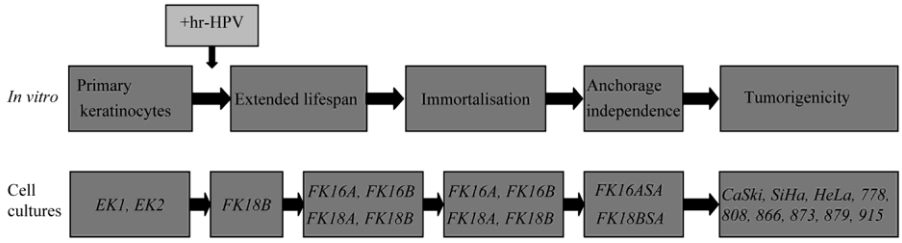
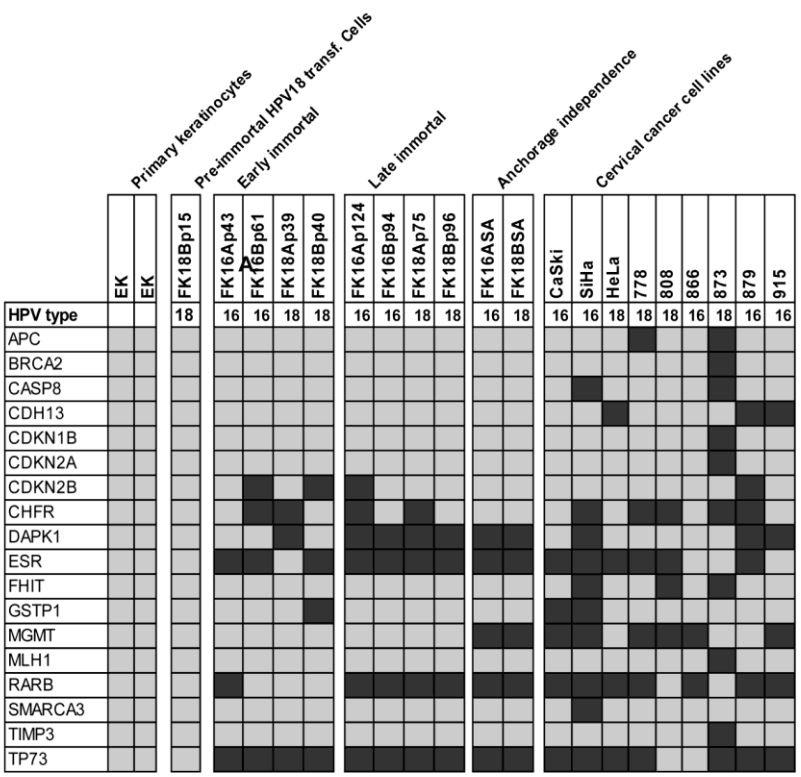


Figure 3. Schematic representation of the multistep process of HPV mediated transformation *in vitro* aligned with the hrHPV transformed keratinocytes and cervical cancer cell lines used in this study.

None of the 29 candidate tumor suppressor genes showed promoter methylation in 2 cultures of primary keratinocytes, nor in the pre-immortal passage of the cell line FK18B (Figure 4). At the early immortal stage, promoter methylation of TP73 was evident in all 4 cell lines and methylation of ESR1 in FK16A,

FK16B, and FK18B. Additive methylation of RARβ and DAPK1 became apparent at later immortal passages of all cell lines. Supplementary methylation of MGMT was associated with anchorage independent growth of FK16A and FK18B. Cervical carcinoma cell lines showed next to markers present in FK cell lines also frequent methylation of CADM1 and CHFR.

A



B

Normal	Early	Late	Intermediate	Late
Normal/pre-immortal	Early immortal	Late immortal	Anchorage ind.	Tumorigenic
	TP73	TP73	TP73	TP73
	ESR1	ESR1	ESR1	ESR1
		RARβ	RARβ	RARβ
		DAPK1	DAPK1	DAPK1*
			MGMT	MGMT
				CADM1
				CHFR
				CDH13*

Figure 4. (A) Summary of MS-MLPA results on primary keratinocytes (EK), HPV transformed cell lines (FK16A, FK16B, FK18A, FK18B; p=passage number) and cervical carcinoma cell lines (CaSki, SiHa, HeLa, 778, 808, 866, 873, 879, 915). Only genes (20/29) that were found to be hypermethylated in at least one of the cell lines are shown. HPV types are listed for each cell line. (B) Longitudinal scheme of epigenetic events associated with hrHPV-mediated transformation as detected in >50% of hrHPV transformed cell lines and cervical carcinoma cell lines. *DAPK1 and CDH13 methylation was detected in 33% of carcinoma cell lines.

In Figure 4A an overview of the MS-MLPA results on all cell lines is shown. In Figure 4B all genes that were found to be methylated in more than 50% of all hrHPV transformed cell lines at the various stages of progression and in cervical carcinoma cell lines are summarized. Notably, all 8 genes that were frequently methylated in the HPV transfected cell lines and cervical cancer cell lines (i.e. TP73, ESR1, RARβ, DAPK1, MGMT, CADM1, CDH13 and CHFR) overlapped with those found to be methylated in the cervical carcinoma specimens.

Confirmation of MS-MPLA results on MGMT promoter by Ms-SNuPE

To confirm MS-MLPA results we selected the MGMT gene for further analysis, since this gene revealed the highest frequency of positive MS-MLPA test results in cervical carcinoma specimens. For this purpose, we used Methylation-specific single-nucleotide primer extension (Ms-SNuPE) analysis, by which methylation differences at specific CpG sites can be assessed in a quantitative manner (46). In this method, DNA is treated with sodium bisulfite followed by amplification of the target sequence using primers specific for bisulfite-converted DNA. The subsequent primer extension reaction utilizes an internal primer which anneals to the PCR product and terminates immediately 5' of the cytosine to be assayed, ^{32}P labelled dCTP and dTTP and *Taq* polymerase. This is followed by denaturing polyacryalamide gel electrophoresis and phosphorimage analysis to quantitate the ratio of C versus T. To confirm our MS-MLPA results, primers were specifically designed to examine the same CpG dinucleotide as analysed by MS-MLPA (nt -459 relative to the transcription start site).

Ms-SNuPE analysis of all cell lines confirmed MS-MLPA results for MGMT in all cases (Figure 5). Cell lines SiHa and CaSki were positive in both settings while the remaining cell lines were negative for MGMT promoter methylation. Also in cervical cancer specimens Ms-SNuPE results were highly comparable to MS-MLPA results (Figure 5). In all except two of the cancer specimens MGMT promoter methylation revealed identical results by MS-MLPA and Ms-SNuPE. The two exceptions were SCC 42 (MS-MLPA positive, Ms-SNuPE negative) and SCC 36 (MS-MLPA negative, Ms-SNuPE positive). It should, however, be noted that in both cases percentages of methylation measured with either or both techniques were near the cut off levels of the assays.

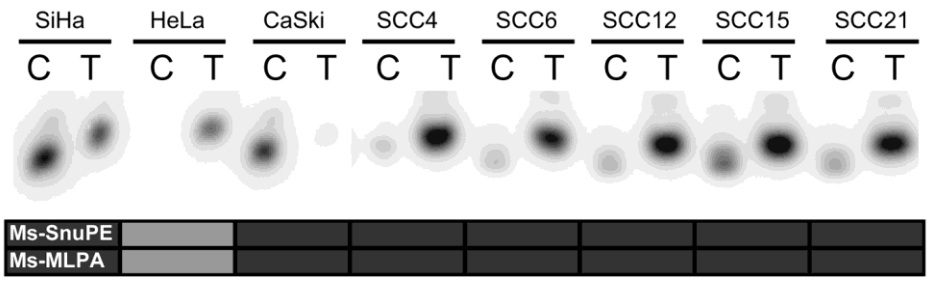


Figure 5. Representative Ms-SNuPE results for MGMT promoter methylation in a subset of cell lines and SCC specimens. Black indicates the sample was methylated and grey indicates the samples were unmethylated. Ms-MLPA results for the same CpG dinucleotide in the MGMT promoter are depicted below for comparison.

Discussion

MS-MLPA analysis of 29 tumor suppressor genes potentially targeted by methylation resulted in the identification of 12 methylated gene promoters in cervical carcinomas, 8 of which could subsequently be associated with consecutive stages of HPV-mediated transformation *in vitro*.

The MGMT promoter was most frequently methylated (i.e. in 92% of carcinomas). Next to the common marker MGMT, also histotype specific markers could be identified. DAPK1 and CADM1 were significantly more frequently methylated in SCCs while methylation of APC, CDKN2B, RASSF1A, TIMP3 and TP73 was significantly more frequent in AdCAs.

The detection of distinct methylation profiles between SCCs and AdCAs is in concordance with literature data, showing higher frequencies of DAPK1 promoter methylation in SCCs compared with AdCAs (47-50, 50-52) and increased rates of APC, RASSF1A and TIMP3 promoter methylation in AdCAs compared with SCCs (50, 53-57).

Based on the analysis of consecutive passages of HPV-transfected keratinocytes we could assign methylation of the different gene promoters as detected in cervical carcinomas to distinct stages of transformation. Moreover, we showed that despite the recent finding that HPV18 E7 targets DNA methyl transferase 1 (DNMT1) involved in de novo methylation (58), none of the 29 genes were found to be methylated in the pre-immortal cells expressing hrHPVE6 and E7. Only following immortalization an accumulation of methylated genes was detected, suggesting that inactivation of these genes is associated with a growth advantage of the hr-HPV containing keratinocytes. To what extent these genes

(except CADM1 (59)) are functionally involved in the different steps during the transformation process remains to be determined.

Methylation of both the TP73 and ESR1 promoter followed by RARβ and DAPK1 promoter methylation were identified as rather early events associated with immortalization. The recent demonstration of an inverse relation between ESR1 protein expression and the severity of the cervical lesion (60) supports our observation of ESR1 promoter methylation being a rather early event during cervical carcinogenesis.

MGMT promoter methylation appeared an intermediate event, which is in line with earlier studies showing MGMT promoter methylation in 26% of invasive cancers and 29% of high grade CIN lesions, compared with only 3% of low grade CIN lesions (61).

Methylation of CHFR, CDH13 and CADM1 was only observed in cervical carcinomas and cervical carcinoma cell lines and can as such be designated as relative late events. To the best of our knowledge this is the first study showing methylation of CHFR to be associated with cervical carcinogenesis. CHFR promoter methylation has, however, been described in other types of cancer, such as breast (62), gastric (63, 64) and colorectal cancer (65, 66).

The detection of CADM1 promoter methylation in cervical cancer cell lines is in concordance with our previous study showing reduced CADM1 mRNA expression associated with promoter methylation in nearly all cervical cancer cell lines, and not in HPV-immortalized cells (67).

Validation of our MS-MLPA results by Ms-SNuPE analysis, an alternative method to specifically assess methylation of a single CpG dinucleotide, confirmed the presence of MGMT methylation (at position -459 relative to the transcription start site), in 92% of carcinomas. The frequency of MGMT methylation as detected by both techniques was different from most previous studies on cervical carcinomas, in which by MSP analysis MGMT methylation frequencies varying from 6.7% to 38% have been reported (68-71). Interestingly, application of the same MSP assay as described in these previous studies, by which methylation at the region from +3 to +137 relative to the transcription start site was analysed, showed evidence for methylation in only 31% of our carcinomas (data not shown). This apparent discrepancy may, at least in part, be explained by heterogeneity of MGMT promoter methylation in cervical carcinomas.

On the other hand, MSP analysis of the cell lines for DAPK1, ESR1, RARß and CADM1 promoter methylation showed a 78-89% concordance between the

MS-MLPA and MSP results (data not shown). The absence of complete concordance may be explained by the fact that MS-MLPA is only based on a single CpG site compared to on average 4-6 CpG sites in MSP assays. Moreover, different CpG dinucleotides were targeted by MS-MLPA and MSP, indicating that, similar to MGMT, in the few discrepant cases heterogeneous methylation patterns may exist within the individual gene promoters.

Taken together, the present data illustrate the significance of our *in vitro* model. In our previous studies we already showed that both morphologically and genetically, the HPV transfected cell lines closely resembled cervical (pre)malignant lesions. (72-75). The current study indicates that also with respect to DNA methylation this model system nicely mimics cervical carcinogenesis *in vivo*. Consequently, it provides a valuable tool to analyse the functional involvement of the identified genes in the respective phenotypical alterations during HPV-induced transformation.

Next to future functional studies, the linkage of the different methylation events to distinct stages of HPV-induced malignant transformation provided first insight in the potential clinical applications of these markers. Early and intermediate methylation events may provide markers for better risk assessment of hrHPV positive women with (ab)normal cytology. On the other hand, late methylation events or a combination of the various events may be used at the time of diagnosis for cancer staging and grading or selection and monitoring of therapy. Future clinical validation studies, for which specific guidelines have been proposed by Pepe et al. (76), on cervical (pre)malignant and cervical scrapes, will ultimately reveal the diagnostic value of the different methylation markers. It should be taken into account that the specificity and sensitivity of the different markers may need to be determined for individual target population as frequencies of promoter methylation can vary amongst different ethnic groups (77, 78).

Gene alterations previously detected in our *in vitro* model system, such as GATA-3, hTERT, MGP and CADM1, were also found in clinical samples (79-82). Therefore, we believe that the methylation events identified in the *in vitro* model system provide potential markers for cervical cancer detection as well, which is underlined by their large overlap with methylated gene promoters found in cervical carcinomas.

In conclusion, MS-MLPA has proven to be a powerful tool to identify genes targeted by DNA methylation in cervical carcinomas. In addition, we were able to link promoter methylation of 8 of the 12 identified markers to distinct stages

of HPV-induced malignant transformation. This resulted in more insight into the natural sequence of methylation events as well as novel candidates for future functional and clinical marker validation studies.

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