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p16 methylation does not affect protein expression in cervical carcinogenesis

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ABSTRACT

Previous studies have reported a frequency range of 19–61% for p16 methylation in cervical cancers. However, p16 is strongly expressed in over 90% of cervical cancers and pre-cancers, due to interactions of HPV oncogenes with p53 and pRb.

In order to clarify these controversial findings, we developed a new bisulphite sequencing protocol to determine the methylation status of p16. DNA extracted from 17 cell lines and 94 microdissected clinical samples was subjected to methylation analysis. p16 expression was confirmed in Western blot and immunohistochemistry.

Complete methylation of p16 was found in none of the dysplastic lesions, but in 26% of the cervical carcinomas. However, immunohistochemistry showed strong p16 expression in all cancers.

These findings indicate that p16 methylation does not implicate loss of p16 expression in HPV-induced tumours. In cervical cancer, methylation of p16 does not seem to be an underlying pathogenic mechanism, but may be a result of increasing genetic and epigenetic instability.

1. Introduction

The cellular tumour suppressor protein p16 has a central function in the regulation of cell cycle activation. It acts as cyclin-dependent kinase (Cdk) inhibitor suppressing Cdk4/6 and Cdk2 by direct binding to the kinase-cyclin complex and arrests the cell cycle in the G1 phase.[1] Loss of p16 function through mutation or hypermethylation initiates phosphorylation of pRb, leading to the release of E2F that activates downstream S phase genes. Conversely, the physiological expression of p16 halts the cell cycle and can lead to irreversible cell senescence.[2]

In many cancers, p16 was found to be inactivated both by mutations and epigenetic modifications. Methylation of a CpG island located in the exon1α of CDKN2A has been found to be associated with a wide variety of malignant tumours, such as NSCLC, colorectal or pancreatic cancer.[3–5] Based on these findings, methylation of the p16 exon1α is considered to be an important and early event in the evolution of squamous cell neoplasias.[6,7]

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Cervical cancer and pre-cancer is characterised by strong overexpression of p16.[8,9] Virtually all cervical carcinomas are induced by carcinogenic human papillomaviruses (HR-HPV) that induce epithelial cell transformation.[10] HPVs are small double-stranded DNA viruses that express two oncogenes, E6 and E7. Their most important interactions are the degradation of p53 by E6 and the disruption of pRb binding to E2F by E7 that results in permanent release of E2F and continuous cell cycle activation.[11] Normally, p16 transcription is blocked by intact pRb/E2F complexes and becomes activated to counterbalance Cdk-induced cell cycle activation.[12] Since in HPV-transformed cells, cell cycle activation is not mediated by Cdks but by E7-related Rb disruption, p16 has no downstream effect. Thus, in HPV-transformed cancers, massive expression of p16 can be found that cannot exert its physiological functions.[8]

The detection of p16 overexpression is used to highlight HPV-associated pre-cancers and cancers. Recently, several studies have shown high sensitivity and specificity of p16-based cytology to detect high-grade cervical intraepithelial neoplasia. In histological and cytological studies, >90% of the HGCIN and cervical cancers strongly express p16[13] so that one would not expect to find methylation of p16 in cervical carcinogenesis.

However, several groups analysed cervical cancers for p16 exon1α methylation and found frequencies ranging from 19% to 61%.[14–21] Most of these studies except for few[17,22,23] show methylation data without any confirmation on the expression level. Two studies have analysed p16 methylation and p16 expression in more detail. In one study unusually low p16 expression frequencies are reported, suggesting that p16 exon1α hypermethylation may lead to loss of p16 expression in cervical carcinogenesis.[23] In contrast, Ivanova and colleagues report a high frequency of p16 expression and only few cases with p16 hypermethylation in their series.

Most data on p16 methylation are obtained using methylation specific PCR (MSP), a method that is based on the conversion of unmethylated Cytosine to Thymidine by bisulphite followed by differential primer systems amplifying either methylated (unconverted) or unmethylated (converted) DNA.[24] Using this approach, reports on methylation are based only on 7 CpGs located in the primer sequences in contrast to >50 CpGs located in the CpG island in p16 exon1α.

In this study, we performed a detailed analysis of p16 exon1α methylation and compared the results to p16 expression analysis. We developed a new nested bisulphite sequencing PCR protocol that allows analysing 28 CpGs located in the p16 exon1α. We have microdissected cervical lesions to reduce the bias of coamplified surrounding normal tissue or stroma. We found composite or complete methylation of p16 exon1α without any influence on the p16 expression indicating that methylation in this region does not suppress p16 expression.

2. Materials and methods

2.1. Cell lines, Western blot

The human cervical carcinoma HeLa, SiHa Caski, C4-1, C33a, ME180, SW756, MS751, MRIH186, MRIH196 and MRIH215, human foreskin keratinocyte (HPK1a and HPK2) and human colorectal carcinoma LS174T, SW48, SW480 and Colo60H cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum and penicillin/streptomycin solution at 37 °C in 5% CO_2. The cervical cancer cell lines MRIH186, MRIH196 and MRIH215 were kindly provided by Elisabeth Schwarz (DKFZ, Heidelberg). All cervical carcinoma cell lines are high-risk HPV positive except for C33a which is HPV-negative but mutated in both RB and TP53.[12]

Cultured cells were lysed in solubilisation buffer containing 20 mM HEPES, 25% Glycerin, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and protease inhibitor. The cell lysates were electrophoresed on 12% polyacrylamide gel and blotted on PVDF membranes. Membranes were blocked with 5% dry milk in TBS-T and incubated with 1 μg/ml of the monoclonal anti-p16 antibody E6H4 (mtm Laboratories, Heidelberg). A HRP-conjugated secondary rabbit–anti-mouse antibody was used at a dilution of 1:5000 and immune complexes were detected using the ECL-Kit (Amersham-Pharmacia, Freiburg).

2.2. Pathological specimens and p16 immunohistochemistry

Seventy paraffin-embedded cervical squamous cell carcinomas (SCC) and 24 CIN lesions were randomly selected in 2003 and 2004 from the Institute of Pathology in Mannheim, Germany. Serial sections of the specimens were generated for H&E staining, p16 staining and DNA extraction. p16 immunohistology was performed using the CINtec histology kit (mtm Laboratories, Heidelberg, Germany) according to the manufacturers' instruction. In brief, after epitope retrieval, slides were incubated with a ready-to-use solution containing the mouse monoclonal antibody E6H4. After incubation with the detection reagent including a HRP-coupled anti-mouse secondary antibody, the staining reaction was performed by adding DAB chromogen. p16 staining was evaluated according to:[9] A diffuse staining of cells (either nuclear or cytoplasmic) originating in the basal layers of the cervical epithelium was considered a positive reaction. Focal staining was considered a negative reaction. Cytospin preparations of the cell lines were stained using the p16 cytology kit (CINTec, mtm Laboratories, Heidelberg) that is based on the same antibodies as the histology kit. Cell lines were considered positive if they displayed nuclear or cytoplasmic p16 staining. As positive control, we included the cervical carcinoma cell line HeLa which strongly overexpresses p16. As negative control, we used the p16-deficient colorectal carcinoma cell line SW480.

2.3. Manual microdissection and DNA extraction

Initially, DNA was extracted from the complete sections of the paraffin-embedded tissue. Subsequently, the pre-cancerous or cancerous tissue of the specimens was microdissected manually from 2 to 4 consecutive 6 μm sections based on the H&E and p16 stained slides. DNA was extracted using the Qiagen DNeasy Tissue kit (Qiagen, Hilden) according to

the manufacturers' instructions, eluted in 30 μl Aqua bidest, used immediately or stored at –20 °C.

2.4. HPV genotyping

HPV detection and genotyping was performed using the Multiplex HPV genotyping Kit according to the manufacturers' instructions (Multimetrix GmbH, Regensburg). In this assay, 24 of the most frequent HPV-types including all carcinogenic types can be detected based on a modified GP5+6+ amplification. After PCR, the fluorescence-labelled bead-bound probes were measured in a Luminex LX100 analyser.

2.5. Bisulphite genomic sequencing

Bisulphite modification of 8 μl DNA was carried using the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturers' recommendations. DNA from HeLa and SW480 cell lines was used as a control and treated concurrently with the samples to monitor the complete conversion of cytosine to thymidine by bisulphite treatment.

A nested BSM-PCR system was developed and performed using the following primer sequences: p16BSM109 for: 5′-GGT TTT TTT AGA GGA TTT GAG GG-3′ and p16BSM652 rev: 5′-ACC TCC TCA TTC CTC TTC CTT AAT T-3′, the nested primers were as follows: p16BSM179 for: 5′-AGG GGT TGG TTG GTT ATT AGA-3′ and p16BSM463 rev: 5′-TAC AAA CCT TCT ACC CAC CTA AAT-3′. The primer sequences were selected not to contain any CpG dinucleotides and to span the typical CpG island in exon1α of the CDKN2A gene analysed by the majority of authors.[24] 50 μl PCR mixes contained 10x PCR buffer, 5 μl 50 mM MgCl2, 0,5 μl 2 mM deoxynucleotide triphosphates, 0.5 mM of each PCR primer (25 pmol/μl), 2.5 U Platinum Taq (Invitrogen) and 2 μl of bisulphite modified DNA. Negative controls without DNA were included in each analysis. Amplification conditions were as follows: initial denaturation at 94 °C for 5 min followed by 40 cycles and 30 cycles for the nested PCR of 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s and finally 72 °C for 4 min. PCR products were electrophoresed and isolated from 2% agarose gels stained with ethidium bromide. Isolated PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden) as recommended.

Direct genomic sequencing of bisulphite modified DNA was performed using the BigDye terminator sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturers' recommendations. The sequencing reactions were analysed on the ABI Prism 310 Genetic Analyser. Methylation PCR products from cell lines were cloned using the TA cloning kit and sequenced subsequently.

2.6. Statistics

Fisher's exact test was used to analyse the association between p16 methylation and p16 expression. Analyses were performed for the complete series as well as restricted to cancers. Methylation status was determined by either complete (including composite methylation) or any methylation in the analysed regions. Two sided *p*-values were used, a *p*-value below 0.05 was considered significant.

3. Results

3.1. Characteristics of analysed samples, HPV detection

In total, 94 cases were analysed for p16 exon1α methylation and p16 expression, including 8 CIN1, 7 CIN2, 9 CIN3, 11 cervical microinvasive SCCs and 59 invasive SCCs of the uterine cervix. The mean age of patients with CIN lesions was 35.9 years, for women with microinvasive cervical tumours and cervical carcinomas the mean age was 41.4 and 52 years, respectively.

All lesions contained high-risk HPV types. The most common HPV types were HPV16 and 18, in addition, HPV 33, 45, 52, 58, 59, 68 and 70 were detected (Table 1).

3.2. p16. Western Blot in cancer cell lines

Western blot analysis showing the p16 protein expression level was performed for sixteen cell lines, 13 cervical cancer and foreskin keratinocyte cell lines and the three colorectal carcinoma cell lines SW480, Colo60H and LS174 that are HPV negative. All HPV-positive cell lines as well as C33a strongly expressed p16 except for ME180, which showed clearly weaker p16 expression in the Western blot. In all the three analysed colorectal cancer cell lines, no p16 expression was detectable (Fig. 1).

3.3. p16. Immunostaining

Cytospins of the cervical cancer cell lines HeLa, SiHa and Caski showed strong p16 immunostaining, whilst in the colorectal cancer cell lines SW480 and SW48, no p16 expression was found. One of the CIN1 cases showed diffuse p16 expression (1/5 CIN1 positive), whilst 3 of 7 CIN2 cases and all of the 9 CIN3 or carcinoma *in situ* cases showed p16 overexpression. All cervical microinvasive carcinomas as well as the cervical carcinoma samples showed strong diffuse p16 expression (Fig. 2).

3.4. p16 methylation analysis in cancer cell lines and clinical specimens

The comprehensive p16 methylation detection approach was evaluated on a series of cervical cancer, HPV-positive foreskin keratinocyte and colorectal cancer cell lines.

Twelve of thirteen high-risk HPV positive or mutated cell lines were completely unmethylated in all 28 explored CpG sites in exon1α. The cervical cancer cell line ME180 appeared as an exception with a composite p16 methylation status displaying both methylated and unmethylated DNA. The colorectal cancer cell lines SW480, SW48 and Colo60H showed full methylation, whereas LS174 was unmethylated except for 4 methylated CpGs.

We found composite or complete methylation of the p16 exon1α in 19.1% (18/94) of the analysed samples, and 25.7% (18/70) of the cancer cases. Initially, complete sections of 29 cervical cancers were subjected to bisulphite genomic sequencing. In this series, 20 were completely unmethylated, 2 of 29 were methylated and 7 of 29 showed a composite methylation pattern, with both methylated and

Table 1 – p16 methylation status in cell lines and cervical specimens

ID	Cell lines/lesions	HPV type	Methylated CpGs	p16 expression	Microdissection
HeLa	Cervical carcinoma CL	18	0	+	na
SiHa	Cervical carcinoma CL	16	0	+	na
Caski	Cervical carcinoma CL	16	0	+	na
C4-1	Cervical carcinoma CL	18	0	+	na
C33a	Cervical carcinoma CL	–	0	+	na
SW756	Cervical carcinoma CL	18	0	+	na
ME180	Cervical carcinoma CL	68	28[a]	+	na
MS751	Cervical carcinoma CL	18	0	+	na
HPK1a	Foreskin keratinocytes	16	0	+	na
HPK2	Foreskin keratinocytes	16	0	+	na
MRIH186	Cervical carcinoma CL	16	0	+	na
MRIH196	Cervical carcinoma CL	16	0	+	na
MRIH215	Cervical carcinoma CL	45	0	+	na
SW480	Colorectal carcinoma CL	–	28	–	na
SW48	Colorectal carcinoma CL	–	28	–	na
Colo60H	Colorectal carcinoma CL	–	28	–	na
LS147T	Colorectal carcinoma CL	–	3, 1[a]	–	na
1	CIN I	16	0	+	–
2	CIN I	na	0	na	–
3	CIN I	16	0	–	–
4	CIN I	16	0	na	–
5	CIN I	16	0	–	–
6	CIN I	16	1	–	–
7	CIN I	16	2	na	–
8	CIN I	16	0	–	–
9	CIN II	na	0	–	–
10	CIN II	16.18	0	–	–
11	CIN II	na	0	–	–
12	CIN II	16	0	–	–
13	CIN II	16	0	+	+
14	CIN II	52	0	+	+
15	CIN II	16	0	+	+
16	CIN III	na	0	+	–
17	CIN III	58	0	+	+
18	CIN III	na	0	+	+
19	CIN III	16	4[a]	+	+
20	CIN III	16	0	+	+
21	CIN III Ca *in situ*	16	0	+	+
22	CIN III Ca *in situ*	na	0	+	+
23	CIN III Ca *in situ*	58.16	0	+	+
24	CIN III Ca *in situ*	16	0	+	+
25	Microinvasive SCC	16	28[a]	+	+
26	Microinvasive SCC	na	28	+	+
27	Microinvasive SCC	16	0	+	+
28	Microinvasive SCC	16	0	+	+
29	Microinvasive SCC	16	0	+	+
30	Microinvasive SCC	33	0	+	+
31	Microinvasive SCC	16	0	+	+
32	Microinvasive SCC	16	0	+	+
33	Microinvasive SCC	16.52	0	+	+
34	Microinvasive SCC	16	28	+	+
35	Microinvasive SCC	16	28	+	+
36	SCC	na	0	+	–
37	SCC	na	28	+	–
38	SCC	16	28	+	–
39	SCC	16	28[a]	+	–
40	SCC	16	28[a]	+	–
41	SCC	na	0	+	–
42	SCC	na	28[a]	+	–
43	SCC	53	0	+	–
44	SCC	16	0	+	–
45	SCC	45	28[a]	+	–
46	SCC	70.16	0	+	–
47	SCC	16.18	28[a]	+	–

(*continued on next page*)

Table 1 – *continued*

ID	Cell lines/lesions	HPV type	Methylated CpGs	p16 expression	Microdissection
48	SCC	16	0	+	–
49	SCC	16	28[a]	+	–
50	SCC	16	0	+	–
51	SCC	16.18	0	+	–
52	SCC	45	28[a]	+	–
53	SCC	16	0	+	–
54	SCC	16.18	0	+	–
55	SCC	18.16	0	+	+
56	SCC	16	0	+	+
57	SCC	16	3[a]	+	+
58	SCC	16	0	+	+
59	SCC	59.16	0	+	+
60	SCC	na	0	+	+
61	SCC	18	0	+	+
62	SCC	16	0	+	+
63	SCC	16	28	+	+
64	SCC	16	0	+	+
65	SCC	16	0	+	+
66	SCC	16	0	+	+
67	SCC	16	0	+	+
68	SCC	16	0	+	+
69	SCC	16	0	+	+
70	SCC	16	0	+	+
71	SCC	16	0	+	+
72	SCC	16	0	+	+
73	SCC	16	28	+	+
74	SCC	16.18	1	+	+
75	SCC	16	0	+	+
76	SCC	16	28[a]	+	+
77	SCC	16	0	+	+
78	SCC	16	28	+	+
79	SCC	16	0	+	+
80	SCC	na	0	+	+
81	SCC	16	0	+	+
82	SCC	16	28	+	+
83	SCC	44	0	+	+
84	SCC	16	0	+	+
85	SCC	16	0	+	–
86	SCC	16	0	+	–
87	SCC	16	0	+	–
88	SCC	16	0	+	–
89	SCC	16	0	+	–
90	SCC	16	0	+	–
91	SCC	16	0	+	–
92	SCC	16	0	+	–
93	SCC	16	0	+	–
94	SCC	16	0	+	–

a Composite methylation pattern of the respective number of CpGs.

unmethylated DNA amplified. In order to exclude the contamination by adjacent normal epithelia and stroma, all further analyses were performed on a microdissected material. Methylation was detected in 3 of 11 microinvasive cervical SCC, whilst one showed a composite methylation pattern, 4 of 30 cervical carcinomas were methylated and one of 30 exhibited a composite p16 methylation pattern, respectively (Table 2, Fig. 3). None of the 24 analysed CIN lesions showed complete p16 exon1α methylation. There was no association between p16 methylation and expression, since all CIN3 and cervical cancer cases showed strong p16 expression irrespective of the exon1α methylation status (Fisher's exact test $p = 0.2$). Restricting the analysis to cancers and analysing partially or only fully methylated regions did not change these findings. Also, there was no association between staining intensity and methylation status (Fig. 2).

Five specimens (2 CIN1, 1 CIN3 and 2 cervical carcinomas) showed isolated methylation of one to four CpGs without any alteration of the p16 expression pattern. Three of them (CIN3 case 19, cervical carcinoma cases 57 and 74) showed methylated CpGs within the classic MSP primer region. This might lead to binding of and subsequent amplification with the

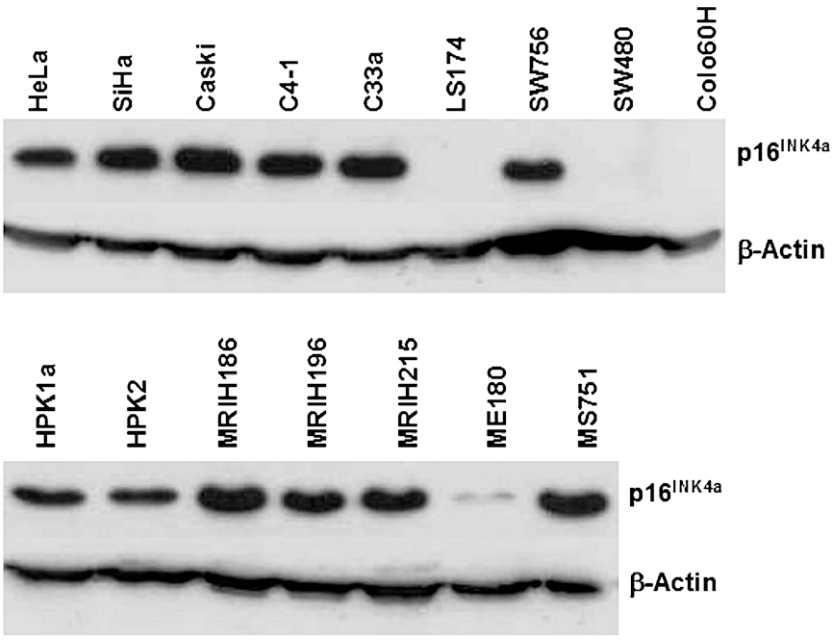

Fig. 1 – p16 Western blot analysis of cervical and colorectal cancer cell lines. The upper signal shows reactivity of the anti-p16 (E6H4 clone) antibody, the lower signal shows the loading control (anti-actin).

methylation specific primers despite the CpG island being largely unmethylated (Fig. 3). We observed no association between patient age at diagnosis or underlying HPV type with the p16 methylation status (Table 1).

4. Discussion

In order to clarify the controversial reports on p16 methylation and p16 protein expression in HPV-related cancers, we developed a new bisulphite sequencing protocol and performed a comprehensive p16 methylation and expression analysis in a series of CIN lesions and cervical cancers. We found 18 of 70 cervical cancer samples with full or composite methylation of the commonly investigated p16 exon1α region. However, in all of these cases, a strong p16 protein expression was observed. Our results confirm previous studies demonstrating a low frequency of methylation in cervical cancer by methylation specific PCR (MSP), yet challenge the conclusions usually drawn from these results.[15,17,20,21] DNA methylation of p16 exon1α can induce activation of S phase genes through Cdk4/6 and Cdk2-mediated phosphorylation of pRb, resulting in the release of E2F and thereby loss of cell cycle control.[25] However, in HPV-related anogenital cancers the p16 inactivation would not confer any further growth promoting effect, because in these cancers the high-risk HPV oncogene E7 induces a permanent release of E2F from its binding to pRb leading to continuous cell cycle activation.

In this study, bisulphite genomic sequencing was performed to get the reliable data on the p16 methylation status of the cervical carcinoma and CIN lesion samples. Reported p16 methylation data are most frequently generated by methylation specific PCR (MSP), an easy and fast approach developed by Herman and colleagues.[24] However, with this assay, only 7 of the 19 CpGs located inside of the 150 bp sequence amplified by MSP can be used to distinguish between the methylated and non-methylated status. In contrast, with our nested PCR system and subsequent bisulphite genomic sequencing we are able to exactly analyse 28 CpGs of the p16 exon1α region. We found full or composite methylation in 25.7% (18/70) of the cervical carcinomas. Nine of 70 (12.9%) cervical carcinomas showed complete methylation, whilst another 9 of 70 (12.9%) showed a composite methylation pattern. Our study particularly showed that after microdissection of the pre-cancerous and cancerous tissue, the percentage of cases with a composite methylation pattern decreased, most probably due to the removal of contaminating normal epithelial and stroma cells. This underlines the importance of extracting the target tissue for methylation as purely as possible. We developed the bisulphite sequencing protocol in order to assess whether the heterogeneous methylation data in literature might be related to partial methylation of p16. Interestingly, our data confirm that the methylation specific PCR system described by Herman is representative of the greater methylation status at this locus. Of the 21 cases that showed any methylated CpGs in the primer binding sites, 18 were completely methylated in the region we analysed and would thus have been picked up by MSP.

The strong expression level of p16 detected by immunohistochemistry has been used to detect HPV-associated precancers and cancers with high sensitivity and specificity.[13] Accordingly, in our study, all high-grade cervical intraepithelial neoplasias and cervical carcinomas showed strong p16 protein expression. Some publications analysing p16 methylation reported lower frequencies of p16 positive lesions.[23,26] However, it remains unclear whether p16 is not expressed in these lesions or if technical problems (e.g. with antigen retrieval) limit its detection.

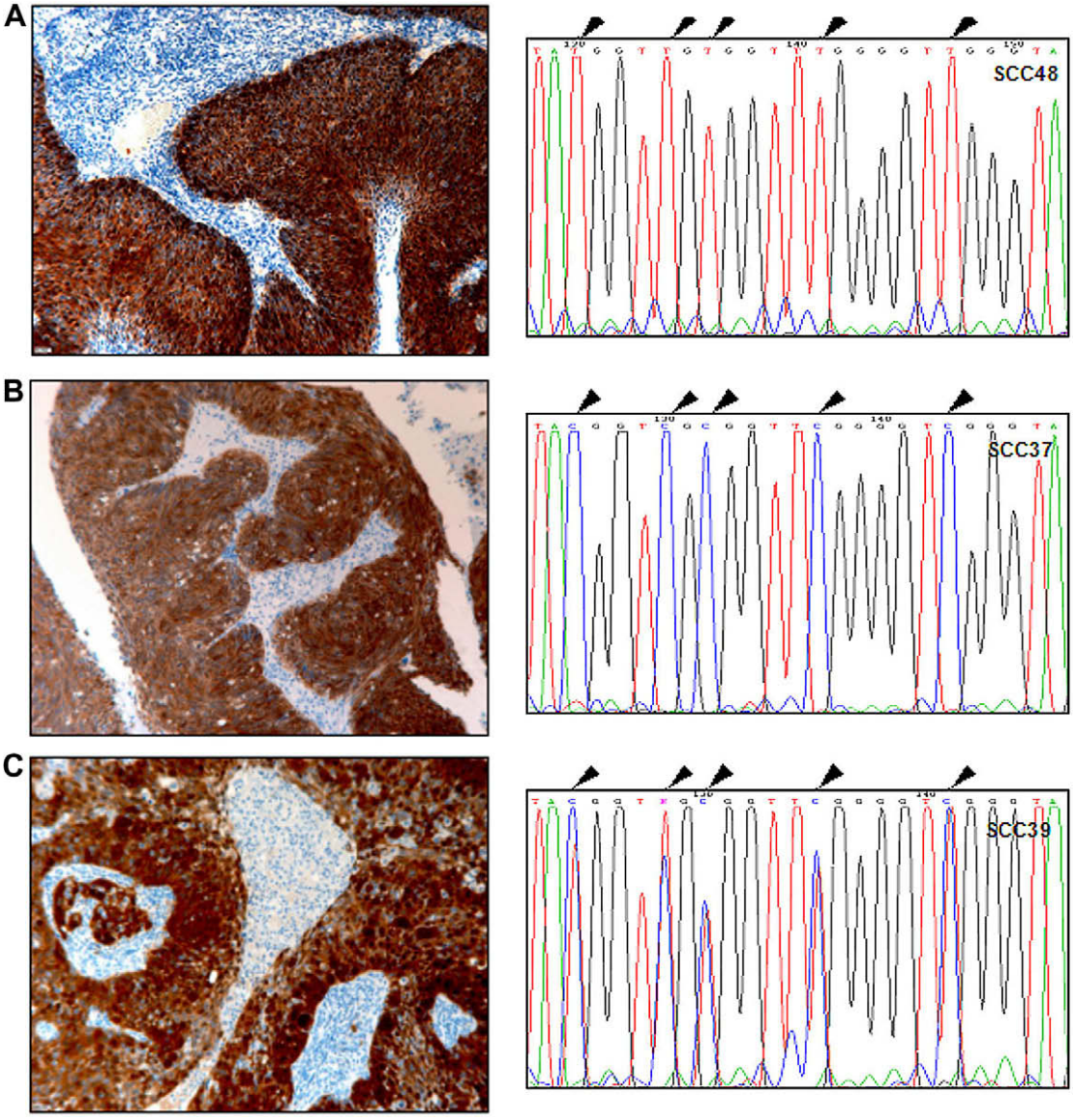

Fig. 2 – Cervical cancer cases with p16 immunohistochemistry and corresponding bisulphite sequencing results. All cancers show strong diffuse nuclear and/or cytoplasmic staining. (A) Squamous cell carcinoma, unmethylated. (B) Squamous cell carcinoma, completely methylated. (C) Squamous cell carcinoma, composite methylation pattern.

Table 2 – HR-HPV, p16 and methylation status in clinical samples

		CIN1	CIN2	CIN3	Microinvasive SCC	SCC
HR-HPV		7/7 (100%)	5/5 (100%)	6/6 (100%)	10/10 (100%)	51/53 (96%)
p16	Pos	1/5 (20%)	3/7 (43%)	9/9 (100%)	11/11 (100%)	59/59 (100%)
Methylation	No	6/8 (75%)	7/7 (100%)	8/9 (89%)	7/11 (64%)	43/59 (73%)
	Single	2/8 (25%)	0/7 (0%)	1/9 (11%)	0/11 (0%)	2/59 (3%)
	Full	0/8 (0%)	0/7 (0%)	0/9 (0%)	4/11 (36%)	14/59 (24%)
Total		8	7	9	11	59

CIN: Cervical Intraepithelial Neoplasia, grades 1–3, SCC: Squamous cell cancer, HR-HPV: Carcinogenic HPV.

In contrast to all other cervical cancer and foreskin keratinocyte cell lines, the high-risk HPV positive cervical carcinoma cell line ME180 showed lower p16 protein expression. Indeed, ME180 displayed a composite methylation status of exon1α with both methylated and unmethylated DNA, a finding that was previously described for HPV-negative tumour types such as NSCLC or colorectal cancer.[3,4]

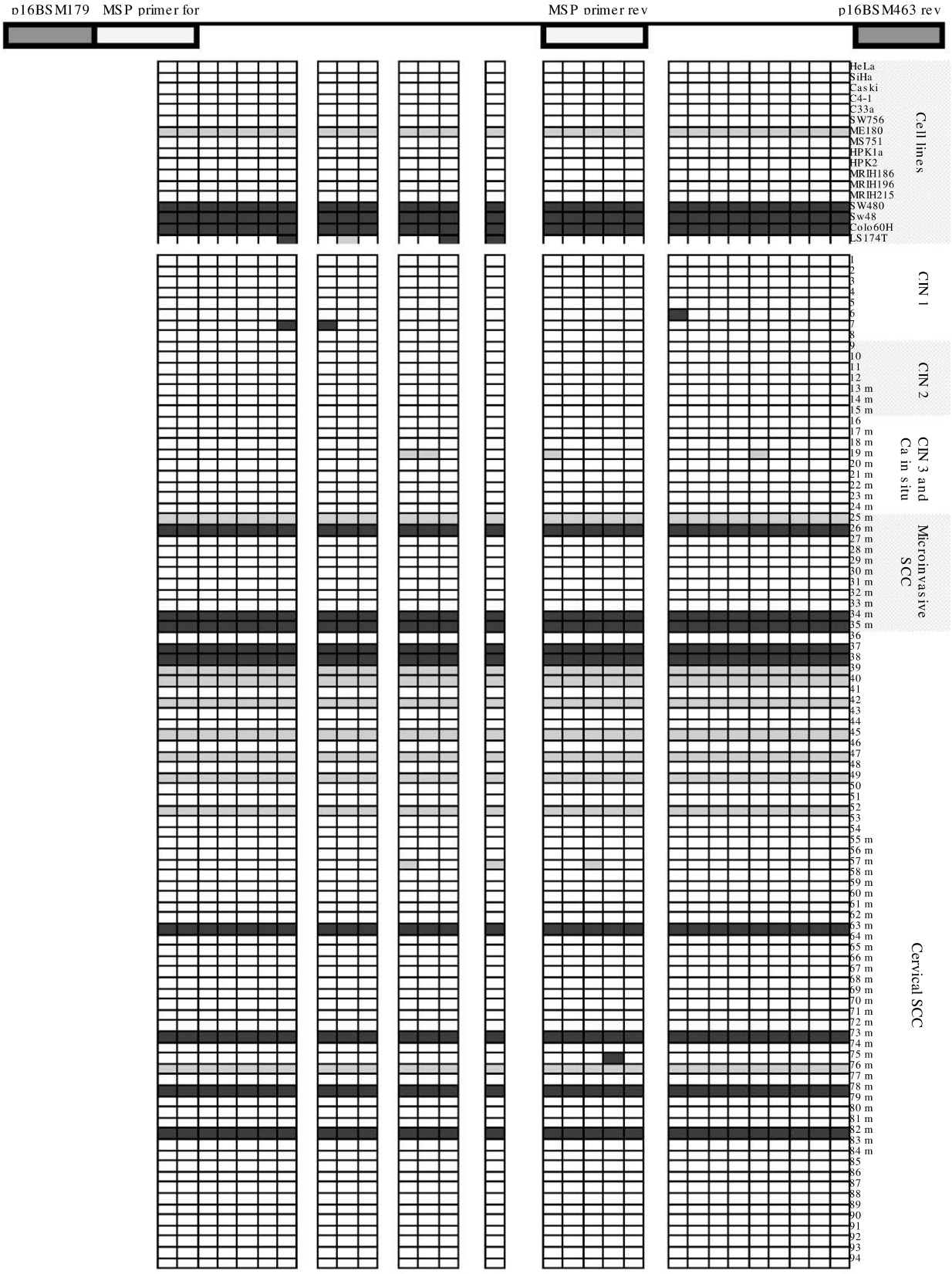

Fig. 3 – Detailed CpG map of p16 exon1α methylation. The CpG map displays the p16 methylation status of every single analysed CpG. White boxes represent unmethylated CpGs, black boxes methylated CpGs, and grey boxes CpGs with composite p16 methylation, respectively. The lower case letter '*m*' represents the manually microdissected cases. The primer positions of our nested BSM-PCR and of the MSP by Herman and colleagues are shown above.

Our data show that p16 methylation in HPV-related high-grade cervical intraepithelial neoplasia and cervical carcinomas does not have any influence on p16 protein expression levels. Many genes have wide-ranging promoter regions and not all CpG islands as well as methylation sites are located in known promoters.[27] The CDKN2A gene locus encodes two different cell cycle regulatory proteins, $p16^{INK4a}$ and $p14^{ARF}$ (Alternative Reading Frame). Unique first exon sequences, exon1α for INK4a and exon1β for ARF, contain promoter elements and are alternatively spliced onto the common exons2 and 3 to create these totally unrelated proteins.[28] Interestingly, exon1α and exon1β are separated by nearly 20 kb of DNA containing several CpG islands. The methylation of one of these CpG islands upstream of exon1α was already described to have an influence on p16 expression.[29] Additionally, there are 36 CpG islands that are spread around CDKN2B and CDKN2A with different methylation patterns. Their influence on the p16 expression level remains to be established. One study describes a differentially methylated region (DMR) for chromosome 9p21, where CDKN2A is located suggesting mono-allelic or parental-specific gene expression in some malignancies.[30]

In cervical cancer, the wide range of p16 methylation frequencies reported and the consistently high p16 expression raise the question whether p16 methylation is rather a result of increasing genetic and epigenetic instability than an underlying pathogenic mechanism. In line with this hypothesis, we only found complete methylation in cancers but not in any of the pre-cancers. In general, our data indicate that caution is necessary when interpreting p16 methylation analyses in the context of cancer progression without any functional data.

Conflict of interest statement

M. von Knebel Doeberitz is a medical advisor to and has financial interest in mtm laboratories that produce an anti-p16 antibody used in this study. There is no other conflict of interest.

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