Methylation status of the E2 binding sites of HPV16 in cervical lesions determined with the Luminex® xMAP™ system

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ARTICLE INFO

ABSTRACT

Cervical carcinogenesis is driven by deregulated E6/E7 expression in dividing cells. A potential deregulating mechanism is methylation of E2 binding sites in the viral long control region, thereby prohibiting HPVE2-mediated transcription regulation. Here the frequency of HPV16E2BS methylation in cervical lesions (SCC, n = 29; CIN3, n = 17) and scrapes (controls, n = 17; CIN3, n = 21) was investigated. Three E2BSs were amplified using methylation independent PCR followed by specific detection of methylated CpGs using the Luminex® xMAP™ system. The frequency of E2BS1, E2BS3 and E2BS4 methylation was significantly higher in SCC compared to CIN3, i.e. 93% vs. 21% (p<0.01), 90% vs. 47% (p<0.01) and 69% vs. 5% (p<0.01), respectively and ranged from 6 to 15% in controls. In scrapings of women with CIN3 methylation ranged from 24 to 33%.
In conclusion, we showed that the MIP–Luminex system is a highly sensitive method for methylation analysis. HPV16 E2BSs methylation appeared highly frequent in SCC, with particularly E2BS3 methylation occurring proportional to severity of cervical disease.

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Introduction

Infection with high-risk human papillomaviruses (hrHPVs) is a necessary cause of cervical cancer. The major hrHPV type associated with cervical cancer is HPV16, accounting for 50% of cervical cancers (Jayshree et al., 2009; zur Hausen, 2002). Viral replication and virion production are coupled to the differentiation program of the infected squamous epithelium and relies entirely on the host cell machinery (Chow and Broker, 1994). Upon differentiation viral transcription, including that of the viral oncogenes E6 and E7, increases and virus assembly occurs in the squamous epithelia undergoing terminal differentiation. Since differentiated cells have already lost the ability to divide, expression of E6 and E7 in differentiated layers does not result in cellular transformation. However, increased expression of E6 and E7 in dividing cells can cause oncogenic transformation, for which interaction of E6 and E7 with the tumor suppressor gene products p53 and pRb, respectively, is essential (Boyer et al., 1996; Scheffner et al., 1990; Steenbergen et al., 2005; zur Hausen, 2002).

Expression of E6 and E7 is regulated by the long control region (LCR) to which cellular transcription factors like Activator Protein 1 (AP1) and specificity factor 1 (Sp1) can bind, as well as the viral protein E2 (Hoppe-Seyler and Butz, 1994). The LCR contains four E2 binding sites (E2BSs) characterized by the specific sequence 5′-ACCN$_6$GGT-3′ (Fig. 1) (Androphy et al., 1987). Binding of E2 to the proximal E2BSs hampers binding of host cell transcription factors to adjacent DNA sequences, such as the TFIID and Sp1 binding sites overlapping E2BS3 and E2BS4, and thereby represses transcription initiation (Tan et al., 1994). Vice versa, displacement of E2 at either one or both E2BSs results in binding of TFIID to the TATA box or Sp1 to the Sp1 binding site leading to transcription activation (Gloss and Bernard, 1990; Tan et al., 1994). On the other hand, binding of E2 to E2BS1 increases viral early gene expression (Dong et al., 1994; Hegde, 2002; Steger and Corbach, 1997), resulting from a stimulation of the function of the transcription factor YY1 binding the YY1-binding site upstream of E2BS1 (Tan et al., 2003), which at this site functions as an activator (O'Connor et al., 1996). The interaction of E2 with E2BS1 is found to be the most stable interaction compared to binding of E2 to the other binding sites, with E2BS2 being the least stable (Sanders and Maitland, 1994). When E2 concentrations are low, E2 binds preferably to E2BS1, leading to E6/E7 expression. As the E2 concentrations increase, the other E2 binding sites will also be bound by E2, resulting in E6/E7 repression (Hegde, 2002). Hence, the observed change in E2 expression during cervical carcinogenesis, with E2 expression being high in low grade cervical intraepithelial neoplasias (CIN1) and decreased in high grade CIN and carcinoma in situ (Bellanger et al., 2011; Maitland et al., 1998; Xue et al., 2010), may at least in part explain the increased E6/E7 expression in high grade lesions.

Accordingly, it has been suggested that deregulated expression of E6/E7 in dividing cells, which is believed to initiate the transformation process may, amongst others, result from loss of E2 repression.
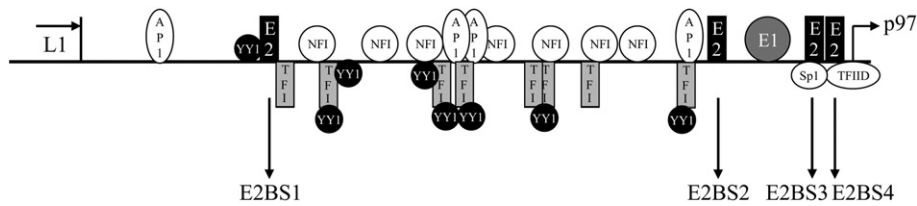
Fig. 1. Schematic representation of the HPV16 LCR. Binding sites of transcription factors (specificity factor 1 (Sp1), Ying Yang 1 (YY1), Transcription Factor I (TFI), Nuclear Factor (NFI), Activator Protein 1 (AP1), Transcription Factor IID (TFIID); boxes and circles) are shown. The LCR contains four E2BSs, with CpG dinucleotides located at nts 7455, 7461 (E2BS1; AC**CG**AATT**CG**GT), 7862 (E2BS2; AC**CG**TTTTGGGT), 37, 43 (E2BS3; AC**CG**AAAT**CG**GT), 52 and 58 (E2BS4; AC**CG**AAAC**CG**GT).

This is substantiated by *in vitro* studies showing a growth repression of cervical cancer cells associated with reduced E6/E7 expression upon E2 overexpression (Dowhanick et al., 1995; Wells et al., 2000). A widely established mode of E2 downregulation is by viral DNA integration into the host cell DNA (Jeon and Lambert, 1995). When integrated, the HPV genome is often disrupted within or just upstream of the E2 open reading frame, thereby either affecting the continuity of that gene or uncoupling it from its promoter sequence (zur Hausen, 2002). However, not all cervical lesions associated with a transforming infection harbor integrated DNA. Recently, it has been reported that only 19% of HPV16 positive CIN3 lesions and 55% of HPV16 positive cervical carcinomas contain integrated viral DNA (Vinokurova et al., 2008). Moreover, previous studies often showed presence of an intact E2 open reading frame in cervical cancers, despite absence of E6/E7 repression (Bhattacharjee and Sengupta, 2006b; Cullen et al., 1991).

This implies that other mechanisms are responsible for the deregulation of E6/E7 in at least part of the cervical precancers and cancers. One such a mechanism may involve DNA methylation, which is a well-known and critical regulator of transcription (Bird, 2002). Each E2BS contains one or two CpG dinucleotides, which are potential targets for DNA methylation. *In vitro* studies have shown that methylation of the CpG dinucleotides in the E2BS consensus sequence inhibits binding of E2 (Thain et al., 1996), and that when E2BSs are methylated E2-mediated transcription regulation is inhibited (Kim et al., 2003). Moreover, the inhibition of E2 binding upon E2BS methylation also affects E1 binding to the origin of viral replication, as its affinity is drastically decreased in the absence of E2 (Dixon et al., 2000; Frattini and Laimins, 1994; Lu et al., 1993; Sun et al., 1996). This implies that E2BS methylation decreases viral replication, thereby contributing to the switch from a productive to a transforming infection.

Several studies have demonstrated that the HPV genome becomes subject to DNA methylation during cervical carcinogenesis, though present data on site specific methylation are inconclusive. Whereas in some studies the LCR and E6 sequences of HPV16 were commonly found to be unmethylated independent of the stage of neoplastic progression, the L1 region was densely methylated in cancers (Badal et al., 2003; Fernandez et al., 2009; Sun et al., 2011). However, in other studies, methylation of the LCR was frequently observed in primary cervical carcinomas, especially at the E2BSs (Bhattacharjee and Sengupta, 2006a; Brandsma et al., 2009).

Most studies in which the methylation status of HPV16 was examined made use of methylation-dependent enzyme cleavage or bisulfite sequencing (Badal et al., 2003; Bhattacharjee and Sengupta, 2006a; Ding et al., 2009; Fernandez et al., 2009; Hublarova et al., 2009; Kim et al., 2003). However, the sensitivity of these techniques may be insufficient to detect low-abundance methylated DNA. Microsphere-based suspension array technologies, such as the Luminex® xMAP™ system, offer a new platform for high-throughput detection of DNA methylation. The Luminex system includes polystyrene microspheres that are internally labeled with two distinct fluorochromes, which can be mixed in various proportions yielding about 100 distinguishable beads (Dunbar, 2006). In the past, bead-based assays have been used predominantly for

quantitative measurements of multiplexed protein–ligand interactions or high-throughput nucleic acid detection through DNA hybridization (Eriksson et al., 2009; Nazarenko et al., 2008).

Here we describe the development of an E2BS methylation detection method based on the Luminex® xMAP™ system. With this method we determined the frequency of HPV16 E2BS methylation in HPV16 positive CIN3 lesions, cervical cancers and scrapes of women with CIN3 compared to HPV16 positive normal cervical samples.

Results

Development of a bead-based detection assay for the analysis of DNA methylation at the E2BSs of HPV16

To determine the methylation status of the E2BSs of HPV16 we developed methylation-independent PCRs (MIPs) followed by a bead-based detection assay using the Luminex® xMAP™ system, as is outlined in Fig. 2. In short, DNAs were first treated with sodium bisulfite, which converts unmethylated cytosines to uracils while leaving methylated cytosines unaffected. This was followed by a MIP amplification with a pair of primers of which one was biotinylated at its 5′-terminus. The amplified products were denatured and hybridized to probes coupled to microspheres, which are either completely complementary to the methylated CpGs (M-probes) or unmethylated CpGs (U-probes). Next, streptavidin–phycoerythrin (SAPE) conjugates were added to bind the hybridized PCR products via streptavidin–biotin binding. Finally, the methylation status was determined based on the fluorescence signal of phycoerythrin (PE) coupled to each microsphere-specific dye.

For amplification of the E2BSs two primer sets were designed, one for E2BS1, and one for E2BS3 and E2BS4 together (see Table 1). Since pilot experiments revealed that the Luminex® xMAP™ system needs at least two nucleotide differences to reliably discriminate between the methylated and unmethylated status (data not shown) and E2BS2 contains only one CpG (AC**CG**TTTTGGGT), E2BS2 was excluded from analysis. The two MIPs (E2BS1 and E2BS3-4) were validated on DNAs isolated from HPV-negative primary foreskin keratinocytes (EKs) and the HPV16 positive cervical cancer cell lines CaSki and SiHa. MIP products were formed for CaSki and SiHa, while no product was seen using EKs (Fig. 3A). Upon sequencing analysis all three binding sites were found to be methylated in CaSki cells, while no methylation was detected in SiHa cells (Fig. 3B). The difference in methylation between these two cell lines most likely relates to the number of copies present, since CaSki cells contain approximately 400–600 copies of HPV16, while SiHa cells only contain 1–2 copies (Fernandez et al., 2009; Schwarz et al., 1985; Yee et al., 1985). Van Tine et al. (2004) reported that only one or a few of the HPV16 copies in CaSki cells showed active transcription, whereas the remaining copies are silenced by methylation. Due to the extreme overrepresentation of methylated HPV16 DNA in CaSki cells, the few copies of unmethylated viral DNA remained undetectable upon bisulfite sequencing analysis.

To accurately determine the analytical sensitivity of the MIP–Luminex detection system we used cloned MIP products of CaSki and SiHa, the sequences of which were confirmed by sequencing
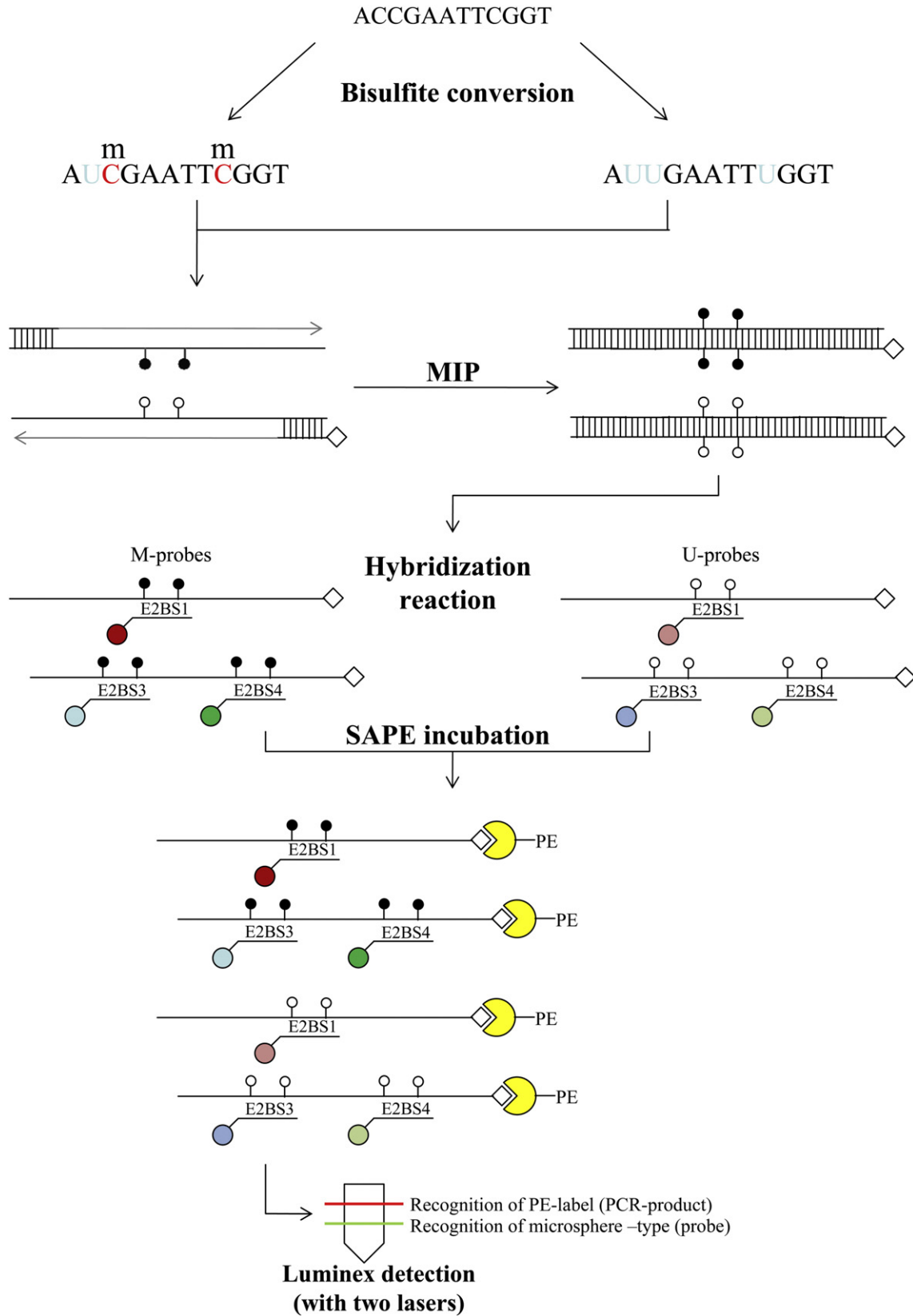
Fig. 2. Principle of detecting methylation with the Luminex® xMAP™ system. Genomic DNA is treated with sodium bisulfite converting unmethylated cytosines into uracils, while leaving methylated cytosines unaffected. Using MIP, PCR products are generated reflecting the same degree of methylation as present in the template (⚲: methylated CpG; ⚲: unmethylated CpG). One of the primers is biotinylated (◊) at the 5′-terminus. Microspheres are coupled to probes specific for fully unmethylated (U-probe) or fully methylated (M-probe) E2BS. Multiplexed hybridization reactions are performed, followed by incubation with SAPE to capture labeled PCR products via streptavidin–biotin binding. The methylation status is determined based on the fluorescence signal of PE (PCR-product) coupled to each microsphere-specific dye (probe).

Table 1

Primer and probe sequences. For luminex the reverse primers were tagged with biotin at their 5′-end.

MIP/Luminex

Target	Sense (5′–3′)	Antisense (5′–3′)	bp	Bead nr.
E2BS1[a]	GTTAG**CG**GTTATTTTGTAGT & GTTAG**TG**GTTATTTTGTAGT	CCATAATTACTAACATAAAAC	94	
E2BS3-4	ATAATTTATGTATAAAATTAAGGG	TACAACTCTATACATAACTAT	156	
ACTB	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	133	
Probes				
BS1 M	TAAT**CG**AATT**CG**GTTGTATGT			45
BS1 U	TAAT**TG**AATT**TG**GTTGTATGTTT			44
BS3 M	**CG**TAAT**CG**AAAT**CG**GTTGAA			24
BS3 U	GG**TG**TAAT**TG**AAAT**TG**GTTGA			23
BS4 M	GAAT**CG**AAAT**CG**GTTAGTATA			89
BS4 U	TTGAAT**TG**AAAT**TG**GTTAGTATA			49

[a] The forward primer for the BS1 region contains a CpG dinucleotide, therefore two primers were designed for this region, one containing a CG in its sequence, while the other contains a TG.

analysis, as positive and negative controls, respectively, for the detection of E2BS methylation in further experiments.

Using these cloned MIP products all probes, i.e. an M-probe and an U-probe for E2BS1, E2BS3, and E2BS4, were found to be highly specific and to accurately discriminate between methylated and unmethylated DNA (Table 2). High median fluorescence intensities (MFI) were observed with all three methylation positive M-probes using CaSki derived MIP products, whereas MFIs for the unmethylated U-probe were negligible. The opposite was seen for SiHa-derived MIP products.

To determine the limit of detection (LOD) of the MIP–Luminex system, CaSki miniprep DNA was spiked with SiHa miniprep DNA in order to obtain a dilution series of $2.5 \cdot 10^6$ copies (100%) to 0 copies

(0%) of methylated E2BSs in unmethylated background DNA and analyzed in triplicate. The LODs were defined as a MFI above the mean background MFI (0%) plus three times the standard deviation. For E2BS1 and E2BS3 the LOD was 0.5% ($1.25 \cdot 10^4$ copies), and for E2BS4 this was 1% ($2.50 \cdot 10^4$ copies) (Fig. 4). Bisulfite sequencing analysis of the same dilution series revealed a LOD for detecting methylated DNA of 10% (data not shown), which is in line with previous reports (Myohanen et al., 1994). This indicates that the MIP–Luminex system has at least a 10 times higher sensitivity for the detection of methylated DNA compared to bisulfite sequencing.

Methylation of BS1, BS3 and BS4 in cervical tissue specimens and scrapings

Using the MIP–Luminex system, methylation of the E2BSs was examined in 19 CIN3 lesions and 29 cervical SCCs containing HPV16. Seventeen scrapings of HPV16 positive women with normal cytology without evidence of cervical (pre)malignant disease up to the next screening round after 5 years served as disease negative controls, as HPV16 positive normal cervical biopsies were insufficiently available. For E2BS1, methylation positivity rates of 21% in CIN3 and 93% in SCC were found. For E2BS3 methylation rates were 47% in CIN3 and 90% in SCC and for E2BS4, these percentages were 5% and 69%, respectively. The E2BS methylation frequencies for E2BS1, E2BS3 and E2BS4 in scrapings with normal cytology were 12%, 6% and 18%, respectively (Fig. 5A). The methylation frequencies for E2BS1, E2BS3 and E2BS4 were significantly higher in cervical SCC specimens, both when compared to controls (p<0.01) and CIN3 (p<0.01). Only for E2BS3 the methylation frequency was found to be significantly higher in CIN3 compared to controls (p<0.01). When combining the methylation results of the three binding sites, all (100%) SCC showed methylation of at least one E2BS, compared to 58% of CIN3 lesions (p<0.01) and 24% of controls (p<0.01). Additionally, the methylation levels of E2BS1, E2BS3 and E2BS4 were significantly higher in SCC compared to CIN3 and normal scrapings (p<0.01) (Figs. 5C–E). Only for E2BS3 the methylation levels were significantly higher in CIN3 compared to normal scrapings (p<0.01).

Next we determined whether E2BS methylation could also be detected in cervical scrapings of women with underlying CIN3. For
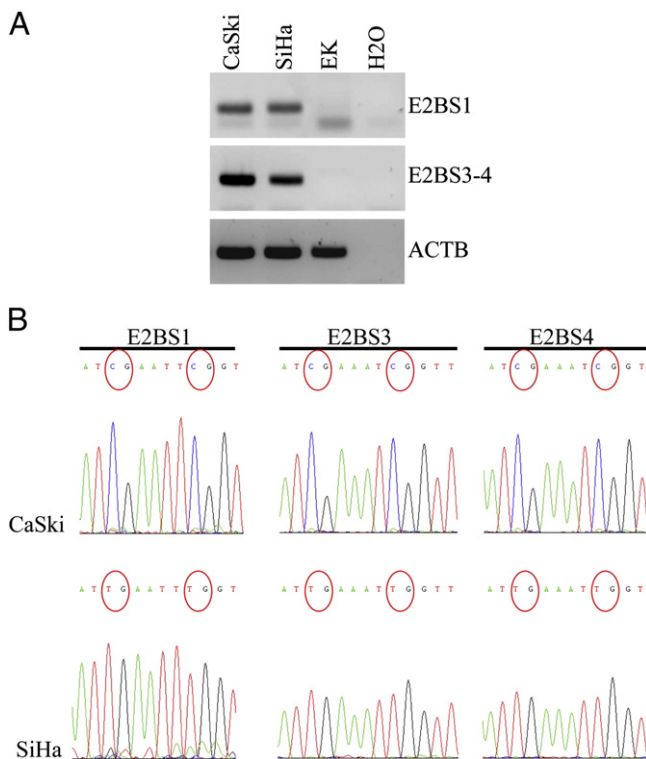
Fig. 3. Methylation status of E2BSs in SiHa and CaSki. (A) PCR products for E2BS1 and E2BS3-4 were formed in CaSki and SiHa. HPV negative primary keratinocytes (EK) showed no E2BS1 and BS3-4 PCR product. The ACTB gene was included as a control for bisulfite treatment. (B) Bisulfite sequencing revealed methylation in CaSki, but no methylation in SiHa at all CpGs of all three E2BSs; CpG/TpGs of each E2BS are circled in red.

Table 2

Summary of the obtained MFI values from the minipreps.

Sample	SQ	E2BS1U	E2BS1M	E2BS3U	E2BS3M	E2BS4U	E2BS4M
		MIP–Luminex results					
CaSki	M	18	2018	45	5699	25	3656
SiHa	U	1617	7	3669	21	3026	19
Blank	–	9	6	7	5	9	10
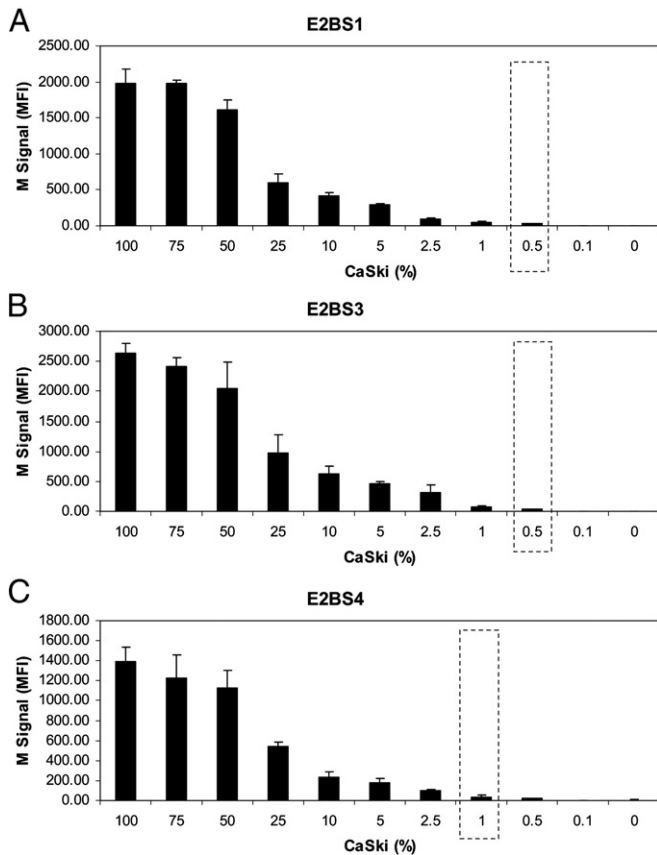
Fig. 4. Limit of detection of the MIP–Luminex system. Results of methylated E2BS1, E2BS3 and E2BS4 using serially diluted CaSki miniprep DNA in a background of SiHa miniprep DNA. For E2BS1 and E2BS3 as low as 0.5% methylated DNA and for E2BS4 as low as 1% methylated DNA can be detected.

this purpose, 21 cervical scrapings of HPV16 positive women with CIN3 were tested for E2BS methylation (Fig. 5B). MIP–Luminex analysis for E2BS1, E2BS3 and E2BS4 methylation showed methylation positivity of 33%, 29% and 24%, respectively. When combining the results, 48% of scrapings were found to be methylation positive for at least one E2BS, which is comparable to any methylation as detected in CIN3 biopsies, i.e. 58%. This finding also implies that the use of normal cytology samples as controls for both tissue specimens and scrapings is unlikely to have a major impact on the results obtained. For E2BS3 and E2BS4 significant differences in methylation levels between normal and CIN3 scrapings were found (p<0.05), which was not seen for E2BS1 (Figs. 5C–E).

Confirmation of MIP–Luminex results by bisulfite sequencing

To confirm the results acquired with the MIP–Luminex, five CIN3 and six SCC tissue specimens were selected for further bisulfite sequencing analysis (Fig. 6). The results of bisulfite sequencing of the cervical tissue specimens were identical to MIP–Luminex results for E2BS1 and E2BS4. In all except two of the CIN3 specimens, E2BS3 methylation revealed identical results by MIP–Luminex analysis and bisulfite sequencing. The two exceptions were methylation positive by MIP–Luminex analysis but negative by bisulfite sequencing, most likely reflecting the differences in LOD between the two techniques. The MIP–Luminex positive, but sequencing negative CIN3 specimens had M values of 38 and 78 MFI (methylation fraction (MF): 2% and 7%), whereas the SCC, which were positive using both techniques, varied from 1111 to 4601 MFI (MF: 48–76%). This indicates that these CIN3 tissue specimens could easily be missed using bisulfite sequencing.

Discussion

Analysis of DNA methylation at three E2BS of HPV16 using the newly developed MIP–Luminex system in cervical tissue biopsies revealed an increase in methylation at E2BS1 and E2BS3 with severity of cervical disease, i.e. 21% and 47% in CIN3 lesions and 93% and 90% in SCC, respectively. The methylation frequency of E2BS3 was significantly lower in normal cytology controls, in which 6% of samples tested positive. Methylation at E2BS4 was low in both normal cytology controls and CIN3 (5–18%), but increased in SCC (69%). When scoring for methylation at any E2BS, 100% of SCCs and 58% of CIN3 lesions were found to be positive, compared to 24% of controls.

The methylation frequencies detected in this study are higher compared to those reported in previous studies using bisulfite sequencing or pyrosequencing (Fernandez et al., 2009; Sun et al., 2011). These observations as well as the discrepancies between published data on HPV DNA methylation may at least in part be related to differences in techniques used. As suggested by Sun et al. (2011), methylated molecules might be overrepresented when using bisulfite treatment followed by cloning and sequencing. In this case the actual methylation pattern might not be correctly reflected, since only a limited number of clones are sequenced. In other studies methylation-sensitive enzyme restriction cleavage was used (Badal et al., 2003; Bhattacharjee and Sengupta, 2006a), which is prone to false positives, due to incomplete digestion or false negatives, due to low abundance DNA methylation (van Vlodrop et al., 2011). With the use of MIP, as applied in the current study, the PCR products analyzed reflect the same degree of methylation as present in the template. Moreover, the specific detection of methylated and unmethylated CpGs by the Luminex® xMAP™ system appeared to be highly specific and sensitive, with a limit of detection of 0.5–1% for E2BS1/E2BS3 and E2BS4, respectively (Fig. 4).

Present findings, showing methylation of all three investigated HPV16 E2BS in CaSki cells and complete absence of methylation in SiHa cells, is in concordance with previous reports (Fernandez et al., 2009; Kalantari et al., 2004; Kalantari et al., 2008; Rajeevan et al., 2006; Sun et al., 2011), and most likely relates to the difference in viral copy numbers between the two cell lines. Methylation of the 400–600 copies in CaSki cells ensures tight expression regulation of E6 and E7 (Fernandez et al., 2009; Kalantari et al., 2008; Van Tine et al., 2004). In these cells it was shown that active viral RNA transcription occurs only at a single-copy or low copy-number site on a derivative of chromosome 14, whereas all other loci were inactive. Transcription from the silent (i.e. methylated) viral DNA copies was activated upon growth in the presence of a DNA methylation inhibitor in a subset of cells (Van Tine et al., 2004), suggestive of additional mechanisms regulating gene silencing. In contrast in SiHa cells, containing only 1–2 copies of HPV16 per cell, viral DNA is found to be integrated within the host cell DNA by interruption of its E2 gene (Baker et al., 1987). Hence interference with E2 binding by E2BS methylation is redundant in SiHa cells. From this it may be inferred that also in cervical high grade lesions and carcinomas which often harbor multiple viral copies (Gravitt et al., 2007; Snijders et al., 2006; Wu et al., 2006), viral transcripts are originating from a single or a few copies only and the remaining copies being silenced by methylation.

In the clinical specimens tested in this study, E2BS1 and E2BS3 revealed the highest methylation rate compared to E2BS4. A recent study by Vinokurova et al. has demonstrated that methylation of E2BS1 results in an increased activity of the p97 promoter in the presence of E2, which intriguingly was associated with binding of a yet uncharacterized protein complex (Vinokurova and von Knebel Doeberitz, 2011). In close proximity to E2BS3, the second most frequently methylated E2BS in SCC, resides a Sp1 binding site and the TATA box (Fig. 1). Whereas in the absence of methylation E2 competes with the transcription factors Sp1 and TFIID for binding to the

Fig. 5. Summary of E2BS MIP–Luminex results in cervical tissue specimens and scrapings. DNA methylation is depicted in black; white boxes indicate unmethylated DNA. (A) The upper section represents cytomorphologically normal scrapings of HPV16 positive women without CIN lesions during 5-year follow-up. The two lower sections represent cervical tissue specimens of HPV16 positive women with CIN3 or SCC. (B) This section represents scrapings classified as moderate dyskaryosis or worse of HPV16 positive women who developed CIN3 within 18 months of follow-up. (C-E) Association between E2BS methylation, disease stage, and sample type. Scatter plots of the levels of E2BS1 (C), E2BS3 (D), and E2BS4 (E) methylation. On the y-axes levels of methylated E2BS are presented; on the x-axes the samples are grouped for each disease stage and sample type. The grey dotted line indicates the cut off value.

promoter (Gloss and Bernard, 1990; Tan et al., 1994), inhibition of E2 binding by E2BS methylation (Thain et al., 1996) induces TFIID and Sp1-mediated transcription activation. Earlier studies have shown that methylation of CpG dinucleotides has little or no effect on the binding of the transcription factors Sp1 (near E2BS3) and YY1 (near E2BS1) (Gaston and Fried, 1995; Holler et al., 1988). However, we cannot rule out the possibility that binding of these transcription sites might be blocked indirectly, either by conformational changes or by interacting with methyl-CpG-binding proteins (Boyes and Bird, 1992; Tazi and Bird, 1990). Taken together, current data indicate that methylation of E2BS1 as well as E2BS3 and E2BS4 results in an increased expression of E6/E7.

In this study, the methylation status of E2BS2 was not examined, due to technical limitations. Previous work done by Kim et al.

(2003) reported the highest methylation rate of E2BS2 compared to the other three E2BSs in a cervical epithelial cell line that was isolated from an HPV16-infected patient. In contrast, in a study on clinical specimens the CpG residue in E2BS2 appeared unmethylated in nearly all CIN and cervical cancer specimens (Piyathilake et al., 2010). Although we were unable to study methylation at E2BS2 using the presently used system, this might be accomplished by the incorporation of a locked nucleic acid (LNA) at the CpG site in the probe sequence. Incorporation of LNAs in oligonucleotides has been shown to improve nucleotide discrimination (Vester and Wengel, 2004).

Previous studies have proposed that testing for HPV DNA methylation may serve as a disease marker for progressive cervical disease (Badal et al., 2003; Ding et al., 2009; Hublarova et al., 2009; Sun et al., 2011). Present findings indicate that also the specific detection

Sample	E2BS1 LU	E2BS1 SQ	E2BS3 LU	E2BS3 SQ	E2BS4 LU	E2BS4 SQ
CaSki	■	■	■	■	■	■
SiHa	□	□	□	□	□	□
CIN3	□	□	□	□	□	□
CIN3	□	□	□	□	□	□
CIN3	□	□	■	□	□	□
CIN3	□	□	■	□	□	□
CIN3	□	□	■	■	□	□
SCC	■	■	■	■	■	■
SCC	□	□	■	■	■	■
SCC	■	■	■	■	■	■
SCC	■	■	■	■	■	■
SCC	■	■	■	■	■	■
SCC	■	■	■	■	■	■

Fig. 6. For comparison, the MIP–Luminex (LU) and bisulfite sequencing (SQ) results are presented for E2BS methylation in CaSki, SiHa, CIN3 and SCC specimens. Black boxes indicate the sample was methylated and white boxes indicate the sample was unmethylated.

of E2BS methylation may be of diagnostic value. Although all SCC showed E2BS methylation at any E2BS, only a subset of CIN3 lesions was methylation positive. Hence, E2BS methylation analysis may serve as a complementary marker to host methylation markers, such as CADM1 and MAL (Overmeer et al., 2008; Overmeer et al., 2009; Overmeer et al., 2010), to improve the early detection of high grade cervical disease. In this respect, E2BS3 methylation seems to be the most promising marker, since only 6% of the normal samples were methylated compared to 47% of CIN3 biopsies.

In this study we showed that the MIP–Luminex system is a highly sensitive and reliable method for the detection of HPV16 E2BS methylation. Hence, this system may in the future be extended further by inclusion of other CpG sites within the HPV-methylome, such as methylation within the L1 region which appears highly predictive for high-grade disease (Sun et al., 2011).

In conclusion, we showed that the MIP–Luminex system is a highly sensitive and reliable method for the detection of methylation at E2BSs. Methylation of HPV16 E2BSs appeared highly frequent in SCCs, indicating that it is a rather late event in cervical carcinogenesis. Particularly methylation of E2BS3 which is most frequent in CIN3 lesions occurs proportional to severity of cervical disease may have diagnostic potential.

Materials and methods

Cell cultures

Primary human foreskin keratinocytes (EKs) and the cervical cancer cell lines CaSki and SiHa were cultured as described previously (Steenbergen et al., 2004).

Cervical samples

This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center. Formalin-fixed, paraffin-embedded (FFPE) samples were collected from patients with CIN3 (n = 19) and cervical SCC (n = 29), which were HPV16 positive as determined by the method described below. The mean age of all women included in this study was 43 years (range 26–74). Per histological group the women had the following mean ages: 37 years (range 26–74) in the CIN3 group and 42 years (range 30–74) in the SCC group. All specimens were collected during the course of routine clinical practice of women who underwent biopsy or surgery and were stored within the tissue bank of the Department of Pathology at the VU University Medical Center. A series of seven consecutive tissue sections were cut from each specimen. The first and last sections (4 μm) were stained with hematoxylin and eosin for histological diagnosis by an expert pathologist. Five in between sections (10 μm) were used for DNA isolation and methylation analysis.

Cervical scrapings were obtained from the population-based cervical screening trial POBASCAM (Bulkmans et al., 2007). We included 17 cervical scrapings of HPV16 positive women with normal cytology without evidence of CIN disease up to the next screening round (after 5 years) and 21 abnormal scrapings of HPV16 positive women with CIN3. The median age of HPV16 positive women with normal cytology was 34 years (range 18–46), and that of the women with abnormal cytology was 35 years (range 24–56).

DNA extraction, HPV typing and bisulfite modification

FFPE tissue sections, cervical scrapings and cell cultures were incubated in proteinase K solution (0.1 mg/ml) for 48 h at 56 °C at 600 rpm. DNA was isolated using the High Pure PCR Template Preparation kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's recommendations. Genomic DNA from cell cultures was isolated with UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (Invitrogen, Breda, The Netherlands).

HPV detection and genotyping was performed using the GP5+/6+− PCR with an enzyme immunoassay (EIA) readout followed by reverse-line-blot analysis of EIA positive cases (Snijders et al., 2005).

For E2BS analysis DNAs were subjected to bisulfite treatment with the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA, USA) according to the manufacturer's recommendations.

Methylation-Independent-Specific PCR (MIP)

Primers used for MIP of E2BS1 and E2BS3-4 are listed in Table 1. In the PCR reaction, 50 ng of bisulfite treated genomic DNA was amplified with FastStart Taq PCR buffer (Roche Diagnostics) supplemented with 2 mM MgCl$_2$, 500 nM of each primer, each dNTP at 200 μM, and 0.75 U of FastStart Taq DNA polymerase in a total volume of 30 μl. Amplification reaction was carried out in a GeneAmp® PCR system 9800 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using the following conditions: 96 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 45 °C or 50 °C, for BS1 or BS3-4, respectively, for 30 s and 72 °C for 45 s, with a final extension of 72 °C for 4 min. MIP products were analyzed by electrophoresis on 2% agarose gels.

Cloning of PCR products

MIP-products of SiHa and CaSki were cloned into pCR®-Blunt II-TOPO vector (Invitrogen, Breda, The Netherlands). Multiple colonies of each construct were grown in selection medium at 37 °C overnight and plasmids were isolated with GeneJET™ Plasmid Miniprep Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's recommendations. The insert of each miniprep was verified by sequencing analysis.

Bisulfite sequencing

To remove unincorporated primers and dNTPs, 2 μl ExoSAP-IT reagent (1:1; USB, High Wycombe, United Kingdom) was added to each MIP product. The mixture was incubated at 37 °C for 15 min, followed by 15 min at 80 °C to inactivate the enzymes. Samples were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI Prism 3130-Avant Genetic Analyzer (Applied Biosystems) according to manufacturer's recommendations. After 30 cycles of amplification, excess dye terminator was removed with the BigDye XTerminator® Purification Kit (Applied Biosystems). Sequences were

analyzed using Chromas Lite (http://www.technelysium.com.au/chromas_lite.html).

Labeling of microspheres with probes

Two probes were designed for each E2BS for Luminex detection (Table 1). Carboxylated microspheres (2.5 · 10^6; Biorad, Veenendaal, The Netherlands) were pelleted in a microcentrifuge for 2 min at 13,000 rpm and supernatant was carefully removed. The microspheres were dissolved in 25 µl MES buffer (0.1 M, pH 4.5; Sigma-Aldrich, Zwijndrecht, The Netherlands), 4 µl of amino-substituted capture probe (100 µM) and 2 µl of a freshly made solution of 100 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (Pierce Thermo Scientific, Rockford, IL, USA). The mixture of microspheres, capture probes and EDAC was vortexed briefly and incubated at room temperature for 30 min at 300 rpm in the dark. After 15 min the reaction was mixed by brief vortexing. A second incubation step was done adding 2 µl EDAC (100 mg/ml). After the coupling reaction, 500 µl of 0.02% Tween 20 (Sigma-Aldrich) was added to the microspheres, followed by centrifugation for 2 min at 13,000 rpm. The supernatant was carefully removed and the coupled microspheres were washed in 500 µl of 0.1% SDS (Sigma-Aldrich) by vortex and centrifuged for 2 min at 13,000 rpm. Finally, the supernatant was removed and the capture-probe conjugated microspheres were resuspended in 100 µl of TE-buffer (10 mM TrisHCl, 1 mM EDTA, pH8) and stored at 4 °C in dark.

Hybridization protocol

To 10 µl of biotinylated DNA target (MIP product), 0.33 µl of conjugated microspheres, 33 µl hybridization buffer (0.15 M TMAC, 75 mM TrisHCl, 6 mM EDTA, 1.5 g/l Sarkosyl, pH8) and 7 µl TE-buffer were added. Hybridization reactions were carried out with an initial step of 95 °C for 3 min, followed by 30 min at 45 °C with shaking speed of 500 rpm. Each sample was transferred into filterplates (multiscreen HTS; Millipore, Bedford, MA, USA) containing 200 µl blocking buffer (0.02% Tween 20). Vacuum filtration was performed for sample collection, followed by an additional washing step with blocking buffer. To each sample 75 µl SAPE (Moss, 1:600) was added and incubated for 15 min at 45 °C with a shaking speed of 500 rpm. Samples were washed three times with blocking buffer and resuspended in 100 µl of blocking buffer. For each experiment, 100 events of each subset of microspheres were analyzed on the Luminex® xMAP™ system (xMAP technology, Austin, TX) to obtain a median fluorescence intensity value (MFI) that was representative of the whole population of each set of beads. The *M* fluorescence signal intensity from hybridization with the M-probe and the *U* signal intensity from hybridization with the U-probe were used to calculate the methylation fraction (MF) with the following formula:

$$MF(\%) = \frac{M}{M+U} \cdot 100\%$$

The 99% confidence interval of the 17 scrapings with normal cytology was used as cut-off value to calculate the percentage of methylation-positive samples.

Statistical analysis

Statistical analysis was performed using SPSS (version 14.0). Associations between methylation frequencies and levels versus histotypes were analyzed using a χ^2-test and Mann–Whitney U-test, respectively. A two-sided p-value of <0.05 was considered statistically significant.

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