Chapter 6

Methylation-mediated repression of PRDM14 contributes to apoptosis evasion in HPV-positive cancers
Chapter 6

**PRDM14 induces apoptosis in HPV-positive cancers**

**Abstract**

Promoter methylation of the transcription factor PRDM14 (PRDI-BF1 and RIZ domain containing 14) represents a common event in human papillomavirus (HPV)-induced cervical cancers and cancer precursor lesions. Here, we aimed to assess the functional consequences of PRDM14 promoter methylation in HPV-induced carcinogenesis.

PRDM14 promoter methylation, expression and consequences of ectopic PRDM14 expression were studied in HPV16-positive cervical and oral cancer cell lines (SiHa, CaSkii and 93VU147T), Human Embryonic Kidney 293 (HEK293T) cells and primary human foreskin keratinocytes (HFK).

PRDM14 mRNA expression was restricted to HEK293T and HFK cells, and could be upregulated in SiHa cells upon DNA methylation inhibition. Ectopic expression of PRDM14 in SiHa, CaSkii and 93VU147T cells resulted in significantly more apoptotic cells, as measured by annexin V labelling, compared to HEK293T and HFK cells. mRNA profiling of 41 apoptosis regulators identified NOXA and PUMA as candidate target genes involved in PRDM14-mediated apoptosis induction. Full-length PRDM14 transactivated both NOXA and PUMA promoters. Transactivation was abolished upon deletion of the PRDM14 DNA binding domain. This suggests that NOXA and PUMA expression is directly regulated by PRDM14, which in case of NOXA was linked to a consensus PRDM14 binding motif in the promoter region.

Taken together, these results suggest that PRDM14 acts as a regulator of NOXA and PUMA-mediated apoptosis induction, thereby providing evidence for a tumour suppressive role in HPV-induced carcinogenesis. The contribution of methylation-mediated gene silencing of PRDM14 to apoptosis evasion in HPV-positive cancer cells provides novel therapeutic options for HPV-induced cancers.

**Introduction**

Cervical cancer is the third most common cancer in women worldwide. Persistent infection with high-risk human papillomavirus (hrHPV) types is causally involved in the development of both squamous cell carcinoma (SCC) and adenocarcinoma (AdCA). These tumours evolve from hrHPV-positive non-invasive precursor lesions, i.e. cervical intraepithelial neoplasia (CIN) and adenocarcinoma *in situ* (ACIS), respectively. Sustained overexpression of the viral oncoproteins E6 and E7 is necessary, but not sufficient, for the development of cervical cancer and its high-grade precursor lesions. This process requires additional (epi)genetic aberrations in host cell DNA. Several studies indicated methylation-mediated repression of tumour suppressor genes to be involved in the process of cervical carcinogenesis. For instance, the gene promoters of CADM1, MAL and hsa-miR-124-2 are frequently methylated in high-grade CIN and cervical carcinomas, which was associated with gene silencing. Re-expression of these genes in HPV-transformed cells suppressed proliferation, anchorage dependent growth, tumourigenicity and/or migration capacity, thereby supporting their tumour suppressive role in cervical carcinogenesis.

In a recent genome wide methylation screen on HPV16-transduced human foreskin keratinocytes we identified the PRDI-BF1 and RIZ domain containing 14 (PRDM14) gene as one of the top methylation targets. PRDM14 encodes a 65 kDa protein belonging to the PRDM transcription-family, whose members can act both as tumour suppressors and oncogenes. PRDM14 promoter methylation was subsequently detected in 98% of cervical SCC and 96% of cervical AdCA, as well as in a major subset of cervical cancer precursor lesions. The observed significant increase in PRDM14 methylation levels with progression of cervical disease, suggests that PRDM14 might play a tumour suppressive role in HPV-induced carcinogenesis.

PRDM14 is a protein, which contains a PRDI-BF1 and RIZ homology (PR)-domain, that is related to the Su(var)3-9, Enhancer of Zeste, Trithorax (SET) methyltransferase domain, and six zinc-fingers that mediate sequence-specific DNA binding. PRDM14 is a critical regulator of cell fate during early mammalian embryogenesis and has been implicated in epigenetic reprogramming. For example, PRDM14 can drive global DNA demethylation in primordial germ cells by downregulation of the DNA methyltransferases (DNMTs) 3A and 3B giving rise to embryonic germ cells. The possible biological relevance of PRDM14 methylation in HPV-induced carcinogenesis is still unknown. Here, we examined the potential functional role of PRDM14 methylation in HPV-induced carcinogenesis.
**Chapter 6**

**PRDM14 induces apoptosis in HPV-positive cancers**

**Materials and Methods**

**Cell culture, DAC treatment and transfections**

Primary human foreskin keratinocytes (HFK) were isolated from foreskin and cultured as described previously. The human cervical carcinoma cell lines SiHa, CaSki and Human Embryonic Kidney 293 (HEK293T) were obtained from the American Type Culture Collection (Manassas, VA, USA). Establishment and culture of the HPV16 positive oral cancer cell line 93VU147T has been described previously. SiHa, CaSki, 93VU147T and HEK293T cells were cultured in Dulbecco’s modified Eagle Medium (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal calf serum, penicillin at 100 U/ml, streptomycin at 100 μg/ml, and l-glutamine at 2 mmol/l (all from Life Technologies). All cell lines were cultured using coated flasks and dishes (Greiner Bio-One, Frickenhausen, Germany).

Incubation of SiHa with 5 μM 5-aza-2′-deoxycytidine (DAC; Sigma Chemical Co, St Louis, MO, USA), was performed as described previously. Transfections were performed using Lipofectamine™ 2000 (Life Technologies) according to the manufacturers recommendations. Human PRDM14 cDNA cloned in the pCMV6-AC-GFP vector and the empty vector pCMV6-AC-GFP were obtained from Origene (Rockville, USA). pCAG-PRDM14, pCAG-deltaDBD and empty vector pCAG were kindly provided by dr. H-H Ng.

**DNA extraction and bisulfite modification**

Genomic DNA from cell lines was extracted by proteinase K digestion followed by standard phenol-chloroform extraction as described previously. DNAs were bisulfite treated using the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA, USA).

**Quantitative MSP (qMSP) analysis**

For the amplification reaction 2.5 μl bisulfite treated DNA (50 ng) was added to 10 μl amplification mix containing 1x Quantitect Probe mix (Qiagen, Leusden, The Netherlands), primers (417 nM each) and probe (208 nM). Sequences of primers and hydrolysis probes are listed in Table 1. Amplification and real-time measurement was performed in the 7500Fast ABI system (Applied Biosystems, Foster City, CA, USA), using the following conditions: 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples were tested in duplicate. Methylation values were normalised to the reference gene β-actin (ACTB) using the comparative Ct method ($2^{-ΔΔCt}$).

**Quantitative RT-PCR**

Total RNA was isolated with TRIzol® reagent (Life Technologies). Two hundred ng RNA was reverse transcribed into cDNA using specific reverse primers with AMV reverse transcriptase (Promega, Leiden, The Netherlands). RT-PCR primer sequences are listed in Table 1. Quantitative RT-PCR (qRT-PCR) was performed in a total reaction volume of 25 μl, containing 25 ng cDNA, 1x TaqMan® Universal PCR Master Mix (Applied Biosystems), 400 nM of each primer and 200 nM probe. Amplification and real-time measurement was performed on the 7900HT Fast ABI system (Applied Biosystems), using the following conditions: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples were tested in duplicate. Universal Human Reference RNA (Stratagene, CA, USA) and reactions without reverse transcriptase were used as positive and negative control.

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**Table 1. Primers and probes sequences for qMSP and qRT-PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers/Probes (5’ - 3’)</th>
<th>Size (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACTB</strong></td>
<td>R: TAACCTCAAGCAATCGCCCA</td>
<td>133</td>
<td>58,2</td>
</tr>
<tr>
<td></td>
<td>P: FAM-GAGCAGCTCAATTAACCGTATA - TAMRA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PRDM14</strong></td>
<td>F: TTTAATCGGGCTTAGACGCTGT</td>
<td>84</td>
<td>59,1</td>
</tr>
<tr>
<td></td>
<td>P: YY - CTTACTCTCGCTTCCAAATTCGAAAATCC - BHQ1</td>
<td></td>
<td>69,1</td>
</tr>
<tr>
<td><strong>snRNP</strong></td>
<td>F: TCTCACCACACTGGGCGA</td>
<td>71</td>
<td>58,8</td>
</tr>
<tr>
<td></td>
<td>P: RTAAAGCTGAGCTGTCTGTGAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NOXA</strong></td>
<td>F: GAAGAGGCGCGCAAGAAC</td>
<td>97</td>
<td>59,7</td>
</tr>
<tr>
<td></td>
<td>P: R: GGACGCACAGCTGGAACGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PUMA</strong></td>
<td>F: GGACACCCTCAAAGCAAAGCGAAGT</td>
<td>90</td>
<td>59,8</td>
</tr>
<tr>
<td></td>
<td>P: ATGAGATGTCGAGGACCCCTCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P: FAM - CTGCTTCTGTTGTCGCGCCT - BHQ1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F: forward; R: reverse; P: hydrolysis probe; YY: Yakima Yellow; BHQ: Black Hole Quencher
to the manufacturers recommendations. Nucleotide substitutions were introduced in the consensus PRDM14 binding site (5’ – GGTCTCTAA – 3’) to generate pGL4.18-NOXA-mut which carried eight mutations (5’ - TGAGAGCC - 3’). Substitutions were confirmed by DNA sequencing. The wild-type (pGL4.18-NOXA) and the mutant reporter construct (pGL4.18-NOXA-mut) were used for measuring promoter activity.

Statistical analysis
Statistical analysis was performed using SPSS (version 20). All experiments were performed in triplicate and mean values ± standard deviation (SD) were calculated. The independent sample t test was used to compare variables. A two-sided p-value of <0.05 was considered statistically significant.

Results
PRDM14 expression is inversely correlated with promoter methylation
In the HPV16-containing cervical cancer cell lines SiHa and CaSki, and the HPV16-positive oral cancer cell line 93VU147T no PRDM14 mRNA was detected. Human Embryonic Kidney 293 (HEK293T) cells and primary human foreskin keratinocytes (HFK) did show PRDM14 mRNA expression, albeit the latter at relatively lower quantities (Figure 1A). QMSP analysis revealed high PRDM14 promoter methylation levels in SiHa, CaSki and 93VU147T cells. Lower levels of PRDM14 promoter methylation levels were found in HEK293T cells, whereas PRDM14 methylation was undetectable in HFK (Figure 1B).

Treatment of SiHa cells with the demethylating agent DAC resulted in a decrease of >45% in PRDM14 promoter methylation and an increase in PRDM14 mRNA expression compared to untreated and mock-treated SiHa cells (PBS) (Figure 1C and 1D). The inverse correlation between PRDM14 methylation levels and PRDM14 mRNA expression suggests that PRDM14 downregulation in SiHa cells is mediated by promoter methylation.

PRDM14 induces apoptosis in HPV16 positive cancer cell lines
To investigate the potential functional role of PRDM14 silencing in HPV-induced carcinogenesis, SiHa, CaSki, 93VU147T, HFK and HEK293T cells were transfected with a PRDM14-GFP fusion construct and a GFP+ control vector. Ectopic PRDM14 expression was confirmed by qRT-PCR (Figure 2A). Upon neomycin selection, all PRDM14 transfected HPV16-positive cancer cell lines showed a strong induction of cell death compared to control transfectants. A strong decline in the number of GFP+ cells was seen in the three cancer cell lines 24, 48 and 72 hours post PRDM14 transfection. In contrast, in control transfectants as well as PRDM14 and
Chapter 6

PRDM14 induces apoptosis in HPV-positive cancers

control-vector transfected HFK and HEK293T cells the number of GFP+ cells 24 to 72 hours post transfection remained the same (Figure 2B-2F). To determine whether cell death could be attributed to apoptosis induction, flow cytometry analysis of living cells double labelled with annexin V and propidium iodide (PI) was performed 24, 48 and 72 hours after transfection. Annexin V/PI+ cells indicate early apoptosis and annexin V/PI- cells indicate late apoptosis. After 24 hours, PRDM14 transfected 93VU147T cells showed significantly (p<0.05) more annexin V+ cells (sum of annexin V+/PI- and annexin V+/PI+) compared to SiHa cells, for which the level was set to 100%. PRDM14 was unmethylated in HFK cells. The level of methylation of PRDM14 in HEK293T cells was approximately 45% lower compared to SiHa cells, for which the level was set to 100%. Methylation levels of untreated SiHa cells were set to 100%.

Figure 1. PRDM14 expression and methylation in SiHa, CaSki, 93VU147T, HEK293T and HFK cells. (A) In none of the HPV16 positive cancer cell lines PRDM14 expression was detected. In HEK293T cells and HFK expression of PRDM14 was detected. (B) SiHa, CaSki and 93VU147T show high levels of PRDM14 methylation. The level of methylation of PRDM14 in HEK293T cells was approximately 45% lower compared to SiHa cells, for which the level was set to 100%. PRDM14 was unmethylated in HFK cells. (C) The PRDM14 methylation levels were reduced by more than 45% in 5000 nM DAC treated SiHa cells. Methylation levels of untreated SiHa cells were set to 100%. (D) Untreated and mock (PBS) treated SiHa cells showed no PRDM14 expression, while SiHa cells treated with 5000 nM DAC expression of PRDM14 was detected.

Figure 2. Ectopic expression of PRDM14 in SiHa, CaSki, 93VU147T, HFK and HEK293T cells. (A) For untransfected SiHa, CaSki and 93VU147T and empty vector (GFP) transfected SiHa, CaSki and 93VU147T cells no PRDM14 mRNA was detectable. SiHa, CaSki and 93VU147T cells transfected with PRDM14 expressed PRDM14. HFK and HEK293T cells did show low expression of PRDM14 and this was greatly increased in PRDM14 transfected cells. The percentage of GFP-positive SiHa (B), CaSki (C) and 93VU147T (D) cells declines in the PRDM14 cells, while that of the empty vector (GFP) transfected and PRDM14 transfected HFK (E) and HEK293T (F) cells remained the same. (G) Flow cytometry analysis of GFP positive SiHa, CaSki, 93VU147T, HFK and HEK293T cells double labelled with annexin V and PI. The amount of apoptotic cells was measured 24, 48 and 72 hours post transfection. The relative annexin V levels were calculated by dividing the amount of annexin V positive cells in the PRDM14 transfected cells, by those with the empty vector transfected cells (* p<0.05).
PRDM14 induces apoptosis in HPV-positive cancers

Chapter 6 PRDM14 induces apoptosis in HPV16-positive cancers

To obtain further insight in the biology underlying PRDM14 induced apoptosis in HPV16-positive cancer cells, mRNA expression levels of 41 apoptosis-regulating genes were analysed by RT-MLPA. Transfected SiHa and HEK293T cells were FACS-sorted and RNA was isolated from the GFP+ cell population. RT-MLPA showed a significant increase in mRNA expression of the pro-apoptotic regulators NOXA (also known as PMAI/P1) and PUMA (also known as BBC3) 48 hours post PRDM14 transfection in SiHa cells (Figure 3A). Increased NOXA and PUMA mRNA levels were not observed in PRDM14 transfected HEK293T compared to control transfecants (Figure 3B). Upregulation of NOXA and PUMA mRNA expression upon PRDM14 overexpression in SiHa cells was confirmed by qRT-PCR analysis (Figure 3C and 3D). These results suggest that PRDM14 induced apoptosis can be ascribed to increased expression of the pro-apoptotic modulators NOXA and PUMA.

PRDM14 induces expression of the pro-apoptotic genes NOXA and PUMA in HPV16-positive cancer cells

To determine whether the putative PRDM14 binding site mediates transactivation of the NOXA promoter by PRDM14, eight single nucleotide substitution mutations were introduced to eliminate its potential recognition by PRDM14 (Figure 4C). Whereas the wild-type NOXA promoter region was strongly transactivated, transactivation of the mutant NOXA promoter by wild-type PRDM14 was largely reduced (Figure 4D). These results demonstrate that NOXA is a direct target for PRDM14 transactivation through a PRDM14 binding site within the NOXA promoter region.
PRDM14 induces apoptosis in HPV-positive cancers

Discussion

The present study showed that DNA methylation of the PRDM14 promoter is inversely correlated with its expression in HPV16-positive cancer cells. Re-expression of PRDM14 in HPV16-positive cancer cell lines induced apoptosis, which could be attributed to a direct upregulation of NOXA and PUMA by PRDM14. In case of NOXA this was mediated through a consensus PRDM14-binding site in its promoter. NOXA and PUMA, also known as PMAIP1 and BBC3, respectively, are pro-apoptotic regulators of the intrinsic apoptosis pathway. These results indicate that methylation-mediated silencing of PRDM14 results in apoptosis-resistance in HPV-transformed cells, pointing to a tumour suppressive role for PRDM14 in HPV-induced cancers.

In contrast, PRDM14 has been indicated as an oncogenic transcriptional activator in human breast cancer and lymphoid leukaemia in mice 28,29. This discrepancy in oncogenic versus tumour suppressive properties in different cancer types is not an uncommon phenomenon. For example, CADM1 functions as a tumour suppressor in several carcinomas such as lung and cervical cancer 30-33, but as oncoprotein in primary adult T-cell leukaemia/lymphoma 34, p16INK4A on the other hand is a tumour suppressor in various cancers, including colorectal and melanomas 35-38, whereas p16INK4A expression appears necessary for the survival of cervical cancer cells 39. These data indicate that oncogenic or tumour suppressive activities are dependent on the cellular context.

The observed tumour suppressive properties of PRDM14 in this study could be dependent on HPV expression. The HPV oncoproteins E6 and E7 interact with multiple proteins, thereby altering the cellular context 39-41. One of the HPV16 E6 targets is CARM1, a type-I arginine histone methyltransferase (HMT) 42. CARM1 methylates histones at p53-responsive promoters resulting in gene activation 43. In addition to directly targeting p53 for degradation through the E6AP pathway, E6 can also target chromatin-bound p53 and repress the activities of the p53 co-activator CARM1. Recently, it has been shown that also PRDM14 can interact with CARM1, thereby regulating chromatin function 44. It may be speculated that the observed PRDM14-mediated upregulation of NOXA and PUMA, both of which are downstream targets of p53, in HPV16-positive cancer cells results from targeting of CARM1 by PRDM14, thereby circumventing E6-mediated CARM1 repression. Next to CARM1, other PRDM14 interacting proteins as well as alternative PRDM14 mRNA transcripts are likely to determine whether or not PRDM14 acts as a transcriptional activator or repressor. Two PRDM14 transcripts have been annotated, one of which encodes the full-length PRDM14 and one short PRDM14 transcript derived from an alternative transcription start-site 45. The latter lacks the PR-domain, which in other PRDM family members has been linked to an oncogenic role (reviewed in 33,34). Recently, an un-annotated shorter isoform of PRDM14 was identified in which exon 2 of PRDM14 is skipped 46. Although the PR-domain (encoded by exon 4-5) is retained in this novel PRDM14 isoform, the activity to induce transcription was abolished. It is presently unclear whether this novel PRDM14 isoform is expressed in cancers, and how the alternative PRDM14 transcripts relate to the tumour suppressive function of PRDM14 in HPV-associated cancers.

Our data suggest that methylation-mediated silencing of PRDM14 represents a novel mechanism of apoptosis evasion, which may contribute to chemoradiation resistance in...
PRDM14 induces apoptosis in HPV-positive cancers

HPV-induced cervical cancers and HPV-positive head-and neck cancers. Cisplatin-based chemotherapy induces apoptosis via the intrinsic pathway and present data indicate a disruption of this pathway by PRDM14 methylation. Additionally, both E6 and E7 of HPV16 are known to contribute to apoptosis evasion by interference with the intrinsic apoptosis pathway as well as the extrinsic pathway via amongst others inhibition of the death inducing signalling complex (DISC formation) 45. A more profound insight into the mechanisms underlying PRDM14-mediated apoptosis regulation in the context of viral oncogene expression is needed to translate current findings into more effective therapeutic strategies for patients with HPV-associated cancers. As epigenetic alterations are reversible, changes in DNA methylation are attractive candidates for cancer therapy 46. Re-expression of PRDM14 may be conferred by treatment with DNA methylation inhibitors such as 5’-aza-2’-deoxycytidine. However, their incorporation into nucleic acids limits their specificity of action, which causes hematologic toxicity. Moreover, their half-life in aqueous solution is very short 47. The use of non-nucleoside DNA methyltransferases (DNMT) inhibitors may offer an alternative approach to reactivate silenced tumour suppressor genes. These inhibitors are not incorporated into DNA and are thought be less toxic and better tolerated in clinical settings. However, the potency of these DNMT inhibitors is much lower than that of 5-aza-2’-deoxycytidine, and for this reason, they have not yet been used in clinics. Combinatorial therapies of non-nucleoside DNMT inhibitors with chemical drugs may offer more effective treatment options 48.

In summary, methylation-mediated silencing of PRDM14 is shown to contribute to apoptosis evasion in HPV-positive oral and cervical cancer cell lines. PRDM14 was identified as a transcriptional regulator of the pro-apoptotic modulators NOXA and PUMA. Given the fact that apoptosis evasion constitutes a major barrier to effective cancer treatment, PRDM14 may serve as a novel therapeutic target in HPV-induced cancers.

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The authors gratefully thank dr. S.M. Wilting, A. Jaspers, L. Moesbergen and N. Hijmering for excellent technical advice and assistance. Moreover, their half-life in aqueous solution is very short 47. The use of non-nucleoside DNA methyltransferases (DNMT) inhibitors may offer an alternative approach to reactivate silenced tumour suppressor genes. These inhibitors are not incorporated into DNA and are thought be less toxic and better tolerated in clinical settings. However, the potency of these DNMT inhibitors is much lower than that of 5-aza-2’-deoxycytidine, and for this reason, they have not yet been used in clinics. Combinatorial therapies of non-nucleoside DNMT inhibitors with chemical drugs may offer more effective treatment options 48. In summary, methylation-mediated silencing of PRDM14 is shown to contribute to apoptosis evasion in HPV-positive oral and cervical cancer cell lines. PRDM14 was identified as a transcriptional regulator of the pro-apoptotic modulators NOXA and PUMA. Given the fact that apoptosis evasion constitutes a major barrier to effective cancer treatment, PRDM14 may serve as a novel therapeutic target in HPV-induced cancers.

References


