Chapter 7

Summary and General discussion
Chapter 7 Summary and General discussion

High-risk human papillomavirus (hrHPV) testing provides superior protection against high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer compared to cytology 1. Therefore, cervical screening by primary hrHPV testing will be introduced in the Netherlands in 2016. However, as only a fraction of hrHPV positive women will have or develop high-grade CIN or worse (CIN3+), novel biomarkers are needed to distinguish hrHPV positive women with high-grade disease from those with clinically irrelevant infections. Biomarkers based on epigenetic silencing of tumour suppressor genes by DNA methylation have shown to be promising. These can be reliably detected in clinician-collected cervical scrapes, liquid based cytology samples and self-collected cervico-vaginal specimens 3,7-9. Methylation of CpG islands within promoter regions of CADM1, MAL and hsa-miR-124-2 is frequently found in hrHPV positive cervical scrapings of women with underlying high-grade CIN 10,11. A combination of CADM1 and MAL methylation markers has shown to be equally discriminatory for CIN3+ as cytology or cytology with HPV16/18 genotyping in hrHPV-positive women in the population-based screening trial POBASCAM 3. To obtain increased specificity and sensitivity rates for CIN3+ a search for novel methylation markers is warranted. Marker discovery by studying epigenetic alterations associated with cervical carcinogenesis may not only yield methylation markers with diagnostic, but also prognostic potential. Moreover, it will improve our understanding of HPV-induced transformation.

In this thesis novel methods for the detection of DNA methylation in both host cell genes and viral DNA were developed. Next to an extended analysis of known methylation events, we focussed on the identification of novel methylation targets, including those that enable the detection of cervical adenocarcinoma (AdCA) and its precursor adenocarcinoma in situ (ACIS). For one hypermethylated gene the potential biological relevance was studied in vitro. Current data suggest that the most optimal sensitivity rates for CIN3+ can only be obtained by using a combination of methylation markers that together target various host genes 3,7-9. However, determining the methylation status of multiple genes in separate assays is time consuming and relatively large amounts of sample material are needed. Multiplexing would enable the analysis of multiple methylation targets and an internal control using a single aliquot of sample material in one assay. This saves material, time, costs and improves quality control. In Chapter 2 we describe a step-by-step protocol to develop a multiplex quantitative Methylation-Specific PCR (qMSP). To accomplish this goal, previously described primers for the methylation targets CADM1, MAL and hsa-miR-124-2 and the reference gene β-actin were modulated to acquire identical annealing temperatures, enabling similar amplification efficiencies for all targets. We compared multiplex TaqMan buffer systems and optimized primer concentrations to allow efficient amplification of all targets. Multiplex qMSP development was analytically validated using dilution series of methylated DNA spiked with unmethylated DNA. Further evaluation on cervical scrapings revealed a high correlation between the obtained normalized ratios of the singleplex qMSPs and the multiplex qMSP. This indicates that multiplex qMSP can be applied to cervical scrapes and used as a triage tool for detection of high-grade cervical lesions in hrHPV-positive women.

Most epigenetic studies on cervical cancer have focused predominantly on squamous cell carcinomas (SCC) and did not include AdCA. Both histotypes display partially distinct epigenetic profiles 10-12. Aiming at identifying methylation events in cervical AdCA we examined in Chapter 3 the methylation frequency of antagonists of the WNT/β-catenin signalling pathway. This pathway known to be active in the majority of colorectal AdCA by means of APC or β-catenin mutations, but has also been found active in cervical cancers by other mechanisms. MSP was used to examine DNA methylation of nine negative modulators of the WNT/β-catenin pathway (APC, AXIN2, DKK3, SFRP2, SFRP4, SFRP5, SOX17, WNTSA and WiFi1) on a small series of cervical tissue specimens, including AdCA and SCC. Four of these genes, i.e. DKK3, SFRP2, SOX17 and WiFi1, revealed frequent methylation (>50%) in AdCAs. Further evaluation using qMSP revealed that the methylation frequencies of DKK3 and SFRP2 were significantly higher in AdCA compared to SCC, i.e. 82% vs. 18% (p<0.01) and 84% vs. 39% (p<0.01), respectively, while SOX17 methylation frequency was significantly higher in SCC than AdCA, i.e. 89% vs. 62% (p<0.05). Methylation of WiFi1 was common in both AdCA (71%) and SCC (54%). Methylation frequencies ranged from 4% to 55% in precursor lesions and from 0% to 5% in normal biopsies. When tested on HPV-positive cervical scrapings, qMSP of the best ACIS/AdCA discriminator genes, i.e. DKK3 and SFRP2, detected all women with underlying ACIS/AdCA, and only 3% of controls. DKK3 and SFRP2 methylation detection could be used to further stratify hrHPV-positive women at risk of AdCA or ACIS and could be helpful for gynaecologists to decide on endocervical curettage or other diagnostic tools, such as a diagnostic cone biopsy.

To determine whether methylation analysis of viral DNA could contribute to CIN3+ detection, we examined the frequency of E2BS methylation of HPV16 in high-grade CIN and SCC. Methylation of these sites is particularly interesting as this may represent a mechanism for deregulated expression of the viral oncogenes E6 and E7 in dividing cells, which is suggested to drive malignant transformation. In Chapter 4 we developed a novel methylation detection method based on Methylation-Independent PCR (MIP) and the Luminex xMAP™ system. With the use of MIP, DNA is amplified regardless of its methylation...
status and by subsequent use of probes coupled to spectrally distinct microspheres the Luminex system can distinguish between the methylated and unmethylated status. This method allows for methylation detection of CpGs within CpG-poor sequences, such as the E2 binding sites (E2BS) in the long control region (LCR) of HPV16. The limit of detection of the MIP-Luminex assay was 0.5-1% of methylated DNA. When combining the methylation results of the three E2 binding sites E2BS1, E2BS3 and E2BS4, all (100%) SCC showed methylation of at least one E2BS, compared to 58% of high-grade CIN and 24% of controls. These results indicate that E2BS methylation is a rather late event in cervical carcinogenesis. When considering the use of E2BS methylation analysis as a complementary marker to host methylation markers, E2BS3 seems to be most promising target, since only 6% of the normal samples were methylated at this site, compared to 47% of high-grade CIN and 90% of SCC biopsies. From a functional perspective, it was interesting that not all lesions with a transforming HPV infection (i.e. CIN3 lesion) show methylation of the E2 binding sites. A possible explanation could be the relationship with the integration status of the HPV genome. In two recently published papers different methylation levels for the E2BSs were measured in samples containing integrated versus episomal HPV DNA. Higher methylation levels were seen in samples with episomal HPV DNA compared to those with single copy integration. In case of multiple integrated viral copies, as seen in CaSkI cells, high methylation levels are associated to silencing of non-actively transcribed copies, thereby fine-tuning E6 and E7 expression levels. It would therefore be interesting to examine whether SCC with high E2BS3 and E2BS4 methylation levels have episomal or multiple copies of integrated HPV DNA, while those with relatively low E2BS3 and E2BS4 methylation levels harbour integrated HPV at single or low copy numbers.

In order to identify novel methylation markers that could contribute to high-grade CIN and cervical cancer detection, we analysed genome-wide DNA methylation alterations associated with HPV-induced cervical carcinogenesis. As described in Chapter 5, we transduced primary human keratinocytes with retroviral HPV16E6E7 and together with their untransduced counterparts they were screened for genome-wide DNA methylation changes at different stages of HPV-induced transformation. With Methylation-specific digital karyotyping (MSDK) 184 locations were identified, which showed differential methylation associated with a transforming hrHPV-infection. Comparison to two public gene expression data sets on cervical carcinomas identified 34 genes with increased methylation in HPV-transformed cells and reduced expression in cervical carcinomas. For 12 genes (CLIC3, CREB3L1, FAM19A4, LFNG, LHX1, MRC2, NNX2-8, NPTX-1, PHACTR3, PRDM14, SOST and TNFSF13) specific methylation in HPV-containing cell lines was confirmed using a real-time MSP detection platform. Subsequent analysis of FAM19A4, LHX1, NNX2-8, NPTX-1, PHACTR3 and PRDM14 in cervical tissue specimens showed increasing methylation levels for all six genes with disease progression. All genes were frequently methylated in cervical carcinomas, with highest frequencies (up to 100%) seen for FAM19A4, PHACTR3 and PRDM14. In Chapter 6, we investigated the functional role of hypermethylation of one of the genes identified in Chapter 5, i.e. PRDM14, in HPV-induced transformation in vitro. Endogenous PRDM14 mRNA was undetectable in HPV16-positive cervical (SiHa, CaSkI) and oral (93VU147T) cancer cell lines, but expression could be upregulated in SiHa cells upon methylation inhibition by 5-aza-2’dexoycytidine treatment. Ectopic expression of PRDM14 in SiHa, CaSkI and 93VU147T cells induced apoptosis, which was linked to a direct upregulation of the pro-apoptotic regulators NOXA and PUMA by PRDM14. Using luciferase reporter assays PRDM14 was found to transactivate both the NOXA and PUMA promoters. Taken together, these data indicate that methylation-mediated silencing of PRDM14 contributes to apoptosis evasion in HPV-induced malignant transformation, pointing towards a tumour suppressive role for PRDM14 in cervical carcinogenesis. Conversely, PRDM14 has been indicated as an oncogenic transcriptional activator in human breast cancer and lymphoid leukemia in mice. As the transcriptional effect for most transcription factors (TFs) is dependent on the joint activity of its binding partners, we screened almost 800 TFs for their ability to interact with PRDM14 using a high-throughput Yeast Two-Hybrid platform (collaboration Prof. B. Deplancke, EPFL, Lausanne, unpublished data). This analysis highlighted two candidate binding partners that both have been implicated in apoptosis regulation in other cancers, but so far have not been studied in relation to HPV-induced carcinogenesis. Moreover, PRDM14 has been described as an inhibitor of DNA methyltransferases (DNMT), 3A and 3B, thereby contributing to epigenetic reprogramming of embryonic cells into a hypomethylated naïve pluripotent state. Since DNMT3B upregulation may contribute to deregulated DNA methylation during cervical carcinogenesis and DNMT3B silencing induces apoptosis in cervical cancer cells, it may be speculated that PRDM14 methylation contributes to HPV-mediated tumorigenesis by altering DNA methylation patterns via DNMT3B derepression, thereby resulting in apoptosis evasion. This however awaits further functional proof.

**Future perspectives**

As outlined above the upcoming era of cervical screening by primary hrHPV testing urges the need for molecular triage markers, with methylation markers being most promising. In addition, methylation markers may also provide useful biomarkers to determine the risk of recurrent CIN2+ after treatment.
The multiplex qMSP described in Chapter 2 and the most promising novel methylation markers described in Chapter 5 are currently being validated in samples collected during several prospective population-based screening trials, such as POBASCAM (Population Based Screening Study Amsterdam), PROHTECT (Protection by Offering HPV Testing on self-sampled Cervicovaginal specimens Trial) and a trial on monitoring women treated for CIN2/3.

In these studies it needs to be taken into account that the frequencies and levels of methylation may differ between cervical tissue specimens, physician-taken cervical scrapings and self-collected cervico-vaginal specimens. This may be the result of distinct levels of background methylation caused by differences in cell type composition combined with differences in analytical sensitivities of the qMSPs for each individual marker. Indeed, a recent evaluation on self-collected lavage samples has revealed that CADM1, which together with MAL is highly informative for cervical scrapes, is less informative for self-samples. For the latter a combination of MAL and hsa-miR-124-2 has emerged as the most informative combination. Ideally, a so-called pan methylation marker panel is constructed which upon multiplexing using the guidelines described in Chapter 2 is applicable to various sample types. Methylation candidates described in Chapter 5 may be helpful for the formation of such a novel multiplex assay. These methylation markers may not only serve as objective detection markers for cervical disease, but may also have implications for the early detection of other HPV positive tumour types, such as anal, head and neck and vulvar cancers.

Interestingly, AdCA specific markers with a 100% sensitivity for AdCA, such as methylated DKK3 and/or SFRP2 as described in Chapter 3, may guide in the future the decision making of the gynaecologist for performing endocervical curettage. In a primary screening scenario with HPV testing, hrHPV-positive women will be referred for colposcopy based on a positive triage test. In case these women show no abnormalities upon colposcopic inspection, a test for AdCA specific markers may be used to indicate whether or not an endocervical curettage or even a diagnostic conisation has to be considered.

Next to host cell gene alterations, methylation of the HPV genome, which increases during disease progression, may serve as a diagnostic marker. A recent study by Kalantari et al showed complementarity between viral and host cell methylation in terms of CIN3+ detection. Although we found that the level and frequency of E2BS3 in the LCR of HPV16 was significantly higher in CIN3+ compared to normal controls, we have no indications that E2BS3 methylation analysis is complementary to host methylation markers, such as CADM1 and MAL. Drawbacks of combined viral and host cell methylation analysis are the need of prior HPV genotyping to study viral methylation and the current absence of a technique which allows the analysis of viral and host methylation in a single reaction.

The development of such a technique would be challenging and require multiple optimisation and clinical validation steps.

In addition to methylation events, other abnormal events associated with HPV-induced carcinogenesis, such as altered microRNA (miRNA) expression may provide alternative or complementary diagnostic markers. Several studies have shown altered miRNA expression profiles in cervical cancers and miRNAs are detectable in cervical scrapes.

Though not as frequently, oncogenic mutations have also been demonstrated in cervical cancers, with PIK3CA mutations being the most common as detected in 38% of SCC and 25% of AdCA. Although with techniques such as whole genome and exome sequencing other and rare mutations can be identified, as recently demonstrated by Ojesina et al, their use in cervical screening programs is presently unclear.

Next to their application as marker for the early detection of HPV-induced cancers, methylation genes described in Chapter 3 and 5 may serve a therapeutic target for treatment of HPV-induced cancers provided their functional relevance is proven. Re-expression of DKK3, SFRP2, WIF1 and PRDM14 inhibits growth of HPV-containing cancer cells. Other candidates described in this thesis still await functional validation. Re-expression may be conferred by treatment with DNA methylation inhibitors such as 5’-aza-2’-deoxycytidine, or by epigenetic engineering/editing for which novel tools are being developed, as has recently been demonstrated for C13ORF18.

Next to their application as marker for the early detection of HPV-induced cancers, methylation genes described in Chapter 3 and 5 may serve a therapeutic target for treatment of HPV-induced cancers provided their functional relevance is proven. Re-expression of DKK3, SFRP2, WIF1 and PRDM14 inhibits growth of HPV-containing cancer cells. Other candidates described in this thesis still await functional validation. Re-expression may be conferred by treatment with DNA methylation inhibitors such as 5’-aza-2’-deoxycytidine, or by epigenetic engineering/editing for which novel tools are being developed, as has recently been demonstrated for C13ORF18.
Chapter 7 Summary and General discussion

REFERENCES


