CHAPTER 8

Summary, General Discussion and Future Perspectives
SUMMARY

Cervical cancer is the fourth most common cancer in women worldwide with highest incidence and mortality rates in low and middle income countries. To decrease the mortality from and incidence of cervical cancer, effective prevention strategies have been developed, i.e. prophylactic human papillomavirus (HPV) vaccination (primary prevention) and cervical screening programmes (secondary prevention). Cervical screening leads to earlier detection and treatment of high-grade cervical intraepithelial neoplasia (CIN2/3) in symptom-free women, resulting in a decrease of cervical cancer. Based on better protection against CIN2/3 and cervical cancer, The Netherlands has recently converted from cytology-based screening to HPV-based screening. In this programme, also HPV testing on self-collected (cervico-)vaginal material (HPV self-sampling) is offered to non-attending women, which has been shown to increase the screening attendance rate. Since many high-risk (hr) HPV infections are transient, a triage test is necessary to identify among hrHPV-positive women those with clinically relevant lesions (CIN2+) and who need referral to the gynaecologist. Accepted triage tests are cytology at baseline combined with repeat cytology at 6-12 months or cytology combined with HPV16/18 genotyping either or not combined with repeat cytology at 6-12 months. However, cytology as triage test has a number of disadvantages. It is subjective and knowing the hrHPV status has been shown to influence the result of the cytology report. When the self-sampled specimen is hrHPV-positive, the woman has to visit the physician for a cervical scrape, since the cytology result on self-sampled material is unreliable. This may result in loss to follow-up of these women. Therefore, other objective, molecular triage tests are needed, which are reliably applicable to both cervical scrapes and self-samples.

In this thesis, we examined molecular host cell alterations which are associated with and contribute to cervical carcinogenesis, and can potentially be useful as biomarkers for risk stratification of hrHPV-positive women. We particularly focused on the biological relevance and diagnostic value of altered DNA methylation, DNA mutations and altered miRNA expression. To this end, different genome-wide discovery screens were performed on in vitro models and clinical specimens. The molecular biomarkers obtained from these and previous screens were tested and validated in independent series of clinical specimens, including cervical tissues, scrapes and self-samples. The biomarkers obtained did not only improve our understanding of the biology of hrHPV-induced cervical carcinogenesis, but also yielded promising molecular markers for cervical screening.

In Chapter 2, we analysed the role of DNA methylation in altered miRNA expression during hrHPV-mediated carcinogenesis. Previously, 34 down-regulated miRNAs were identified in CIN2/3 and cervical cancer. Downregulation of two miRNAs could be explained by a chromosomal loss of the gene locus. Another six miRNAs (miR-149, miR-203, miR-210, miR-375, miR-572 and miR-638) were located within a CpG island, suggesting that these may be targeted by DNA methylation-mediated silencing. To test this hypothesis, methylation specific PCRs (MSP) targeting their CpG-rich promoter regions were developed. Analysis of an in vitro model of hrHPV-immortalised keratinocytes and cervical cancer cell lines revealed increased DNA methylation of miR-149, -203 and -375 with progression to malignancy. Moreover, expression levels were restored after treatment with a demethylating agent. These results indicate that the reduced expression levels of the three miRNAs result from DNA methylation of their promoter sequences. Furthermore, DNA methylation of miR-203 and -375 was significantly increased in CIN3 and cervical cancer compared with controls in tissue specimens. Similar to previous observations on miR-375, ectopic expression of miR-203 in cervical cancer cells resulted in a decreased proliferation rate and reduction of anchorage independent growth, indicating a tumour suppressive role for miR-203 in hrHPV-mediated transformation. Additionally, we showed that increased DNA methylation of miR-203 in CIN3 compared with controls was also detectable in a pilot series of hrHPV-positive cervical scrapes. Therefore, methylated miRNAs may provide putative biomarkers for detection of CIN3 and cervical cancer.

Besides methylated miRNAs, we also aimed to obtain more insight in the occurrence of DNA mutations during cervical carcinogenesis. Although DNA mutations have been comprehensively analysed in cervical cancers, the mutation profile in CIN lesions is largely unknown. In Chapter 3, we profiled tissue biopsies of CIN2/3 and cervical cancer for the presence of somatic hotspot mutations in cancer-related genes by next generation sequencing (NGS). PIK3CA exon 9 was the most frequently mutated gene locus in cervical cancer and the only mutated gene locus detected in CIN2/3. These findings were verified in a large, independent series of tissue specimens (n = 647) covering all stages of cervical carcinogenesis using high-resolution melting-guided Sanger sequencing. The PIK3CA exon 9 mutation frequency was 37.1% in cervical cancer and 14.3% in squamous cell carcinoma (SCC) and 14.3% in adenocarcinoma (AdCA), 2.4% in CIN3 and 0% in CIN2, CIN1 and normal cervix. Analysis of an additional tissue series of 46 CIN2/3 lesions with a known 5-year history of preceding hrHPV infection (PHI), used as a surrogate for duration of lesion existence, revealed PIK3CA exon 9 mutations in only 5.4% of so-called advanced CIN2/3 with a PHI ≥ 5 years. No mutations were detected in CIN2/3 with a PHI < 5 years, also referred to as...
early CIN2/3. This finding suggests that somatic mutations in PIK3CA exon 9 represent a relatively late event during cervical carcinogenesis compared with DNA methylation and copy number alterations (CNA), both of which already become apparent in a major subset of CIN2/3. In line with this, we observed a low variant allele frequency (VAF) of PIK3CA exon 9 mutations in cervical cancer, suggesting that this mutation is only present in subclones of the tumour. Interestingly, most PIK3CA exon 9 mutations were hotspot mutations p.E542K (c.1624G>A) and p.E545K (c.1633G>A). Both nucleotide substitutions correspond to C>T (G>A on opposing strand) mutations at a TCW (W = A or T) trinucleotide motif, which is associated with a mutational signature by apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC). APOBEC family members are known to play a role in the host defence response to hrHPV infections, and several mechanisms why hrHPV infections would benefit from activation of APOBEC enzymes, in particular APOBEC3, have recently been suggested (reviewed in 27). APOBEC3 activation has been linked to the induction of both mutations in the viral genome, explaining the wide diversity of HPV variants, and in the host cell genome, which is suggested to contribute to the carcinogenic progression of hrHPV-induced transforming lesions28,29. Recent whole-exome sequencing analysis on 228 cervical carcinomas by The Cancer Genome Atlas (TCGA) revealed 14 mutated genes, including PIK3CA, EP300, FBXW7, PTEN and HLA-A30. In line with our results, PIK3CA was detected as the most frequently mutated gene (26%) in cervical cancer and the overall mutation signature strongly correlated with APOBEC-mediated mutagenesis.

In Chapter 4, we performed an unbiased genome-wide DNA methylation screen using methyl binding domain (MBD) protein-enriched DNA sequencing (MBD-Seq) on both in vitro and patient-derived samples to identify novel biologically relevant DNA methylation markers. We selected the top 20 methylated genes for further comprehensive verification analyses by multiplex targeted bisulphite NGS and MSP. This resulted in the identification of 3 DNA methylation markers, GHSR, SST and ZIC1. Validation analysis by multiplex qMSP revealed that the DNA methylation levels of all 3 genes increased significantly with passaging of hrHPV-immortalised keratinocytes and with severity of CIN lesions to cervical cancer. The clinical performance of the 3 methylated genes in hrHPV-positive cervical scrapes (n = 220) was evaluated by logistic regression analysis. The AUC for CIN3+ (CIN3 plus cervical cancer) detection varied from 0.86 to 0.89, showing a good and similar performance for all 3 DNA methylation markers. The group of CIN2/3 however represents a heterogeneous disease with a various risk for progression to cancer and a variable duration of lesion existence. As also discussed above, previous studies have shown that a subset of CIN2/3 display a mutational signature by apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC). APOBEC family members are known to play a role in the host defence response to hrHPV infections, and several mechanisms why hrHPV infections would benefit from activation of APOBEC enzymes, in particular APOBEC3, have recently been suggested (reviewed in 27). APOBEC3 activation has been linked to the induction of both mutations in the viral genome, explaining the wide diversity of HPV variants, and in the host cell genome, which is suggested to contribute to the carcinogenic progression of hrHPV-induced transforming lesions28,29. Recent whole-exome sequencing analysis on 228 cervical carcinomas by The Cancer Genome Atlas (TCGA) revealed 14 mutated genes, including PIK3CA, EP300, FBXW7, PTEN and HLA-A30. In line with our results, PIK3CA was detected as the most frequently mutated gene (26%) in cervical cancer and the overall mutation signature strongly correlated with APOBEC-mediated mutagenesis.

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Previous research has shown that DNA methylation markers originally discovered in tissue specimens and evaluated in cervical scrapes do not necessarily show a good clinical performance in self-samples, due to a likely lower amount of disease-related or “indicator” cells30. Therefore, we set out to identify the most informative DNA methylation markers for direct application on hrHPV-positive self-samples in Chapter 5 by performing a genome-wide DNA methylation screen (Infinium 450K array) on self-sampled material. This discovery screen revealed 12 DNA methylation markers for CIN3 detection that were subsequently analysed by multiplex qMSP in large series of hrHPV-positive lavage (n = 245) and brush self-samples (n = 246). The qMSP data were analysed by logistic regression analysis and resulted in a 3-gene methylation classifier (ASCL1, LHXB and ST6GALNAC5) with optimal performance. Validation of this 3-gene methylation classifier in large independent self-sample series from a screening cohort of non-attendees (n = 199 lavage self-samples and n = 287 brush self-
samples) showed an excellent and reproducible clinical performance for CIN3 detection in both hrHPV-positive lavage (AUC = 0.88; sensitivity = 74%; specificity = 79%) and brush (AUC = 0.90; sensitivity = 88%; specificity = 81%) self-samples. In CIN2, 50% of both lavage and brush self-samples was detected using the DNA methylation classifier. Importantly, all self-samples from women with cervical cancer, both squamous cell carcinoma (SCC) and adenocarcinoma (AdCA), scored DNA methylation-positive. From these data it may be concluded that the 3-gene methylation classifier, identified by genome-wide DNA methylation profiling, is highly effective for direct triage on hrHPV-positive self-samples.

In Chapter 6, we performed a systematic analysis of 12 previously identified methylated genes by qMSP to compare their DNA methylation onset and patterns during hrHPV-induced carcinogenesis using an in vitro model and clinical specimens. First, we analysed hrHPV-transformed cell lines, which showed that the onset of DNA methylation occurred either at the early immortal stage (ANKRD18C, FAM19A4, GHSR, JAM3, PRDM14, SST and ZSCAN1) or at the late immortal stage (C13orf18, EPB41L3, PHACTR3, SOX1 and ZIC1). All 12 genes showed gradually increased DNA methylation levels towards tumorigenic cells. Subsequently, we analysed a series of hrHPV-positive cervical scrapes (n = 113) to compare the DNA methylation patterns of all 12 genes in clinical specimens. The majority of genes displayed a comparable DNA methylation pattern with significantly increased DNA methylation levels associated with progression to cervical cancer. Based on the DNA methylation profile of all 12 genes in cancers, a cancer-like methylation-high pattern was determined. This pattern was also observed in 72% of CIN3 and 55% of CIN2, whereas the other subset of CIN3 and CIN2 lesions displayed a methylation-low profile similar to hrHPV-positive controls. The presence of a cancer-like methylation-high pattern in a subset of women with CIN2 and CIN3 lesions suggests a higher risk of progression to cervical cancer.

To study whether other molecular events associated with cervical carcinogenesis may provide alternative or complementary triage markers, the marker potential of miRNAs was studied in Chapter 7. We performed genome-wide small RNA sequencing (sRNA-Seq) on 74 hrHPV-positive self-samples to identify a panel of deregulated miRNAs that can predict the presence of CIN3 and cervical cancer in self-sampled material. This discovery screen yielded a miRNA panel of 9 miRNAs for detection of CIN3 with a combined AUC of 0.89. Validation of 5 miRNAs (let-7b, miR-15b, miR-20a, miR-93 and miR-222) by qPCR resulted in a combined AUC of 0.78 for CIN3+ detection. This data shows that deregulated miRNA expression associated with CIN3 and cervical cancer development can be detected in hrHPV-positive self-samples and offers a novel molecular triage strategy applicable to self-samples for detection of CIN3 and cervical cancer. Of note, we applied a different method for validation compared with the discovery (qPCR versus sRNA-Seq), which could explain the discrepancy between the observed AUCs.

In conclusion, in this thesis, we showed that increased DNA methylation of protein-coding genes and miRNAs as well as altered miRNA expression is associated with cervical cancer development and becomes detectable at the stage of precancerous disease in tissue specimens, cervical scrapes and self-samples, and is most prominent at the invasive cancer stage. Three genome-wide discovery screens yielded novel promising DNA methylation and miRNA markers for detection of CIN2/3 and cervical cancer in cervical scrapes and self-samples. Increased DNA methylation is common to all cancers and a major subset of CIN2/3 lesions, in particular CIN2/3 lesions with a PHI of ≥ 5 year that are likely the clinically most relevant lesions with the highest short-term progression risk to cancer. DNA mutations on the other hand were found to become detectable at a later stage and are mostly associated with cervical cancers. The molecular findings described in this thesis are illustrated in Fig. 1.

### Figure 1. hrHPV-mediated cervical carcinogenesis, including a schematic representation of the molecular host cell alterations described in this thesis. The colour change from yellow to blue represents the increase in molecular host cell changes. Adapted from Steenbergen et al. Normal: normal cervix; Productive CIN: CIN1/2; Transforming CIN: CIN2/3.
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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The findings reported in this thesis support a transition to triage hrHPV-positive women by molecular markers. Yet, several aspects warrant further research to fully elucidate the molecular mechanisms driving cervical carcinogenesis following a transforming hrHPV infection, and to implement these findings in HPV-based cervical screening programmes and risk stratification.

Comparison and integration of molecular data

As described in the Summary (Fig. 1), we have identified some of the important molecular alterations associated with cervical carcinogenesis, which encompass both genetic and epigenetic events. Although all molecular events increase with progression from CIN to cancer, for most of them it is currently unclear whether and how the molecular alterations relate to each other in time. Hence, future genome-wide data integration as well as studies on the biological relevance of these events will be important to fully unravel the multistep pathway of hrHPV-induced carcinogenesis.

Previous research on CNA in cervical cancer and CIN2/3 revealed that a subset of CIN2/3 lesions had a cancer-like CNA profile [24]. In line with this, a cancer-like methylation-high pattern was also observed in a subset of CIN2/3 lesions (Chapter 6). Our studies on CIN2/3 lesions and corresponding cervical scrapes with a known duration of lesion existence (represented by duration of ≥ and < 5 years PHI) show that the subset of CIN2/3 with a cancer-like molecular profile correspond to the advanced lesions with a PHI ≥ 5 years (Chapter 4 and 24–26). These data strongly suggest that high DNA methylation levels and many CNA are indicative of a higher short-term cancer risk. In contrast, it is still unknown how altered miRNA levels are reflected in the heterogeneous CIN2/3 lesions. Since various miRNAs are deregulated by CNA or DNA methylation (Chapter 2 and 25–26), altered miRNA profiles occur most likely together with CNA and DNA methylation cancer-like profiles. Importantly, further insight into molecular alterations and whether they are able to predict which CIN2/3 lesions will progress or regress, may help to reduce over-referral and overtreatment.

It would be interesting to evaluate whether specimens with a cancer-like CNA profile cluster together with a cancer-like methylation-high pattern. Accordingly, CNA analysis on genome-wide DNA methylation profiles would be of interest. Unfortunately, we were unable to acquire information on copy numbers from our MBD-Seq data due to varying read-counts between samples (Chapter 4). CNA analysis on our Infinium 450K array data from self-sampled material (Chapter 5) revealed increased CNA in self-samples from women with SCC compared with the CNA-silent profiles in self-samples from control women (unpublished results). These CNA included known gains such as chromosome 1 and 3q. Regrettably, CNA were not detectable in self-samples from women with CIN3, likely due to the low abundance of lesional cells in these specimens.

In contrast to the classical Knudson two-hit hypothesis that DNA methylation of a tumour suppressor gene promoter on one allele is accompanied by a loss of the second allele [25], we made an opposite observation. As described in Chapter 4, we found an association between DNA methylation of GHSR, SST and ZIC1 and gain of 3q (see Summary Chapter 4 above). Interestingly, also two of the genes in the 3-gene methylation classifier developed for self-samples (Chapter 5) are located on a known gained region in cervical cancer, i.e. LHX8 and ST6GALNAC5 both on 1p31.1. It remains to be determined whether their DNA methylation levels are also increased in the presence of a 1p gain. Moreover, it is currently unknown which molecular event occurs first and drives the other molecular alteration. Further integrated copy number and DNA methylation studies in longitudinal in vitro models are warranted to investigate whether DNA methylation is a response to CNA or a cause of CNA due to genomic destabilisation.

Previous research showed that all cervical cancers display high CNA and DNA methylation levels [22,23]. Recently, TCGA published the most extensive molecular characterisation of 228 primary cervical cancers performed to date [24,45]. The complete TCGA dataset including CNA, DNA methylation, mRNA and miRNA data is publicly available and can be used for comparison or integration with own datasets. Comprehensive integration analysis using the various genome-wide molecular datasets, resulted in three molecular clusters, which distinguishes the AdCa cluster from two SCC clusters (keratin-high and keratin-low) and highlights the molecular heterogeneity within cervical cancer. Comparison of separate molecular datasets showed that the cervical cancers with a CNA-high profile do not overlap with a DNA methylation-high profile. This indicates that cervical carcinomas may at least in part have a different molecular background, suggesting that the multistep pathway of cervical carcinogenesis may vary. Of note, specific local overlapping CNA, i.e. losses and gains, and increased DNA methylation levels were not described and may still be present. Furthermore, miRNA expression profiles were evaluated showing among others significant lower expression levels of miR-203a in the keratin-low cluster of SCC compared with the keratin-high cluster. The AdCa cluster was characterised by high expression of miR-375...
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Clinical implications

Dna methylation of host cell genes

The epigenetic host cell alterations in CIN2/3 and cervical cancer that were evaluated in this thesis may become effective molecular markers for detection of clinically relevant lesions in HPV-based cervical screening programmes. Given increased interest in self-sampling, molecular markers with optimal performance in self-sampled material are of highest importance and most promising. Our genome-wide DNA methylation discovery screen on self-sampled material revealed a 3-gene methylation classifier applicable to self-samples in cervical screening with an excellent sensitivity for CIN3 (74-88%) and cervical cancer (100%) in both lavage and brush self-samples at a specificity of 80% (Chapter 5). This classifier also showed a good clinical performance compared with other previously reported comprehensively evaluated molecular triage methods in self-samples, such as the DNA methylation panel FAM19A4/miR-124-2 and HPV16/18 genotyping. Future validation studies in large series of clinical material are needed to confirm this.

Presently, triage of hrHPV-positive women is done by repeat cytology in the Dutch revised cervical screening programme. Women with hrHPV-positive self-samples need an additional visit to a physician for taking a cervical scrape, because cytology triage on self-sampled specimens is unreliable. With the development of molecular markers, especially the DNA methylation markers described in this thesis, full molecular cervical screening on self-samples can be envisioned (Fig. 2). Molecular triage is objective and directly applicable to self-samples (Chapter 5), which allows for a fast diagnostic track and reduces loss to follow-up. DNA methylation markers particularly detect cervical cancer and the clinically relevant advanced CIN2/3 lesions (Chapter 4 and 23,25,26), i.e. CIN2/3 associated with a preceding hrHPV infection ≥ 5 years. These lesions with a cancer-like methylation-high pattern (Chapter 6) are considered to have an expected high short-term risk of progression to cervical cancer. As a consequence, only hrHPV-positive women with a positive DNA methylation test may need referral to the gynaecologist and subsequent treatment. HRHPV-positive, DNA methylation-negative women could be offered a repeat hrHPV test at 12-18 months, and referred in case of a positive test at that occasion. This would identify women with a persistent infection and reveal most of the remaining clinically relevant CIN2+ cases. This approach can be particularly beneficial for women at childbearing age, knowing to have many regressing CIN lesions. The use of a DNA methylation assay in this age category can prevent overtreatment and related morbidity, such as cervical insufficiency and preterm delivery. Notably, cytology may miss cervical cancer and advanced CIN2/3, while it detects cellular abnormalities associated with early CIN2/3 (Fig. 2).
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To implement the 3-gene methylation classifier as molecular triage test, future research remains necessary. Since our studies used self-samples from Dutch screening cohorts of non-attendees, further (prospective) validation studies in an attending screening population and other international cohorts are warranted. Similar as recently performed for DNA methylation panel FAM19A4/miR-124-2, a long term evaluation study in a screening cohort of hrHPV-positive women is necessary, which will yield the (longitudinal) negative predictive value of the 3-gene methylation classifier as triage test. Also, further insight in molecular host cell alterations with respect to the progression risk of CIN2/3 lesions is required. Presently, a prospective study is ongoing, investigating whether the DNA methylation markers FAM19A4 and miR124-2 in hrHPV-positive women harbouring CIN2 or small CIN3 lesions is associated with progression of these lesions (CONCERVE trial). The findings in this clinical study will elucidate whether DNA methylation marker-negative or positive CIN lesions are indeed predictive for respectively regression or progression of CIN lesions. Furthermore, at the technology level improvements are necessary. For widespread implementation of DNA methylation analysis in screening, assay simplification, automation and cost reduction are needed. In addition, education of health care professionals is important to make them comfortable with the use of DNA methylation tests in clinical decision making, and to disseminate this knowledge to the women target population.

Finally, detection of the host cell DNA methylation markers such as ASCL1 and LHX8 might serve as primary screenings markers. Such approach may improve the diagnostic track with respect to time and costs, since only one test is necessary. Although the data concerning the prevalence of these markers in the general population and sensitivity and specificity for advanced CIN2/3 lesions are promising, more data on both cervical scrapes and self-samples are needed before implementation in cervical screening can be realised.

miRNA expression analysis

Although the clinical performance of the miRNA signature (Chapter 7) is not yet as good as observed for DNA methylation markers (Chapter 5), miRNAs could serve as a valuable addition to other molecular biomarkers. Preliminary analysis of the DNA methylation and miRNA markers identified in Chapter 5 and 7 revealed an improved clinical performance for CIN3 and cervical cancer detection upon combining both marker types compared with each marker type separately (unpublished data). Therefore, further research using a combined approach of different complementary molecular markers in one panel, (which is expected to be technically challenging) may even yield a better molecular triage classifier than those in this thesis.

Therapeutic targets

Besides using the genetic and epigenetic host cell alterations that were evaluated in this thesis for early detection and risk stratification, they may also serve as therapeutic targets. Further research on DNA methylation inhibitors (i.e. 5’-aza-2’deoxycytidine) or other epigenetic engineering tools targeting methylated genes may provide potential novel treatment options. For treatment, it is however important that off-target effects, such as activation of oncogenes, are reduced to a minimal level. This is one of the big challenges in future research on using these kind of treatment agents53.

Alternatively, the identified PIK3CA DNA mutations in cervical cancer (Chapter 3 and TCGA data30) may be of interest as therapeutic target, since PI3K/AKT/mTOR inhibitors are available. To date, only a few clinical trials showed data on using these agents as a targeted therapy in cervical cancer patients34. These results are promising, but further clinical trials are warranted. It has been suggested that these inhibitors may also improve the effect of immunotherapy in patients with cancer harbouring PIK3CA mutations35.
Furthermore, the overall high mutation load in cervical cancer may make this cancer type vulnerable to immune checkpoint disruption. The association between mutational burden and response to immune checkpoint therapy is documented in non-small cell lung cancer and melanoma. Amplification of PD-L1 and PD-L2 has also been detected, providing a potential immunotherapeutic target for a subset of cervical cancers. Other immunotherapeutic treatment strategies for cervical cancer are currently under evaluation, including agents which stimulate the immune response against hrHPV-transformed cells.

REFERENCES


