Summary, discussion and future perspectives
Infection with high-risk human papillomavirus (hrHPV) is necessary for the development of cervical cancer and the virus can be found in virtually all carcinomas.\(^1\) However, although persistent hrHPV infection can result in a precancerous lesion, additional genetic and epigenetic aberrations within the host cell genome are required for the development of invasive carcinoma. Two to 3 years after infection high-grade cervical intraepithelial neoplasia (CIN, CIN2/3) may develop.\(^2,3\) From this state it takes an additional 10-30 years for progression towards carcinoma.\(^4\) Due to this long interval it is possible to timely detect and treat the precancerous lesions, preventing carcinoma. In current screening programs this is accomplished by cytological assessment of cervical scrapes, the Papanicolaou (Pap) test. However, this test suffers particularly from a high proportion of both false-positive and false-negative results.\(^5,6\) Testing for the presence of HPV greatly improves the sensitivity for the detection of CIN2+, yet is less specific as also transient infections with negligible risk of malignant progression are detected.\(^7,8\) As in the near future hrHPV-testing is expected to become the primary cervical cancer screening tool it is imperative to have objective biomarkers enabling risk-stratification of hrHPV-positive women. The most suitable biomarkers are those reflecting the aberrations underlying development of high-grade CIN and invasive carcinomas (CIN2+).

Carcinomas display a multitude of aberrations, both functionally relevant ones, so-called drivers, as well as passenger aberrations without any biological significance that have accumulated during the long term period of progressive premalignant disease. Since the genomic chaos is likely to be less prominent in CIN2/3, investigation of aberrations in these lesions may facilitate identification of functionally relevant events. Several studies have investigated aberrations in CIN2/3 (summarised in Chapter 1), yet the reported results vary, indicating the heterogeneous nature of these lesions. In this thesis the heterogeneity of CIN2/3 was investigated at both the chromosomal and epigenetic level. The potential contribution of factors such as the duration of preceding hrHPV infection as well as the hrHPV type present was examined.

**Chromosomal heterogeneity of CIN3 is related to duration of preceding hrHPV infection**

In Chapter 2 high-resolution arrayCGH was performed on microdissected formalin-fixed paraffin-embedded (FFPE) cervical biopsies. These included 6 CIN1, 11 CIN3 with a short-term (<5 years) preceding hrHPV infection (PHI), 24 CIN3 with a long-term (≥5 years) preceding hrHPV infection and 6 squamous cell carcinomas (SCC) and adjacent CIN3. Unsupervised hierarchical clustering analysis revealed that 81.8% of CIN3<5yrPHI showed no or hardly any chromosomal aberrations versus 33.3% of CIN3≥5yrPHI. The percentage of array oligonucleotides deviating from the normal state, further addressed as the number of aberrations, increased per lesion grade from 0.16% in CIN1, to 2.83% in CIN3<5yrPHI, 16.52% in CIN3≥5yrPHI, 28.75% in CIN3 adjacent to SCC and 34.27% in SCC. CIN3≥5yrPHI had significantly more chromosomal aberrations compared to CIN3<5yrPHI and these aberrations were similar to those detected in
cancers. This may imply that these lesions have a high short-term progression risk and require immediate treatment. CIN3<5yrPHI, on the other hand, could be managed by close surveillance as they are likely to have low short-term risk of progression. This may be particularly beneficial to women of reproductive age, as treatment of CIN2/3 coincides with some degree of morbidity of the cervix and can give rise to preterm delivery.\(^9\)

Non-random gained regions on chromosomes 3q, 1p and 1q were indicated to be early events in the host cell genome, followed by gain on chromosome 20 and loss on chromosome 2q. Interestingly, some of these gained regions contain genes that have been reported to have increased expression in cervical carcinoma as result of a copy number gain, for instance DTX3L and MCM2, both located at 3q.\(^{10,11}\) Therefore these chromosomal aberrations, associated with advanced high-grade CIN, may provide candidate biomarkers, being either DNA-, RNA- or protein-based, for triage of hrHPV-positive women and as such warrant further investigation. Moreover, the presence of specific chromosomal aberrations allows future discrimination between CIN2+ with low and high short-term progression risk, which will be of particular importance for validation studies on other candidate biomarkers, such as methylation markers, for risk-assessment of hrHPV-positive women.

**Chromosomal heterogeneity of CIN2/3 is partly related to the hrHPV type present**

Since different hrHPV types confer variable risks of CIN2/3 and carcinoma\(^{12,13}\), it was investigated in Chapter 3 whether the extent and type of chromosomal aberrations in CIN2/3 is also dependent on hrHPV type. Using high-resolution arrayCGH, the number and pattern of chromosomal aberrations of 43 p16\(^{INK4a}\)-immunopositive (FFPE) CIN2/3 with ≥5yrPHI were related to the hrHPV type present. Sixteen of these lesions harboured HPV16, 3 HPV18, 14 HPV31, 1 HPV33, 4 HPV45, 1 HPV51, 2 HPV52 and 2 HPV58. Unsupervised hierarchical clustering analysis revealed 87.5% of lesions with HPV16 to have relatively few aberrations. The presence of relatively few aberrations in HPV16-positive lesions could be validated in an independent series of previous cross-sectional collected CIN2/3.\(^{14}\)

The ‘number of aberrations’ in lesions with HPV16 was lower (i.e. 11.4%), though not significantly, than in lesions with non-HPV16 types (HPV\(^{non16}\), 16.1%) and HPV31 (18.3%). Direct comparison of lesions with HPV16 versus those with HPV31 revealed losses at chromosomes 2q, 4p, 4q, 6p, 6q, 8q and 17p and gains at 1p and 1q to be significantly more frequent in HPV31-positive lesions (FDR<0.2). Pathway analysis on genes encoded by these differentially affected regions indicated the top three canonical pathways to be involved in antigen presentation, allograft rejection signaling and cytotoxic T-lymphocyte-mediated apoptosis of target cells. The majority of the genes associated with these pathways included major histocompatibility complex genes located at chromosome 6p and, as such, may contribute to immune evasion. Interestingly, also the p53 locus at chromosome 17 was significantly more affected in lesions with HPV31 compared to HPV16, though it remains uncertain to what extent p53 activity is affected in
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these cases. Of note is that, although not significant, lesions with HPV16 had the highest incidence of 3q gain compared to HPV\textsubscript{non16} or HPV31-positive lesions. This aberration is highly frequent and consistently detected in cervical carcinoma\textsuperscript{15-21} and has been suggested to predict progression of CIN.\textsuperscript{22} Genes located within this region, as was recently shown for PIK3CA23, may have an important role in malignant transformation.

Analysis of focal aberrations as an approach to identify relevant driver genes underlying disease development

To further define genes affected by chromosomal aberrations that may drive malignant transformation, focal aberrations were investigated in Chapter 4. In previous chapters the focus was on chromosomal regions that were frequently affected (≥25% in the data set), or that significantly differed between lesions with different HPV types and the recurrent chromosomal aberrations were still relatively large. Observed with a lower frequency in these data sets were the so-called focal aberrations (<3 Mb in size). Analysis of focal aberrations may facilitate the identification and validation of potential driver genes in the carcinogenic process, as due to their small size they harbour only few genes.

Using a newly developed software tool, the set of 60 CIN2/3 described in the previous two chapters were analysed for focal aberrations. A total of 235 recurrent focal aberrations were detected, of which 74 genomic locations harbouring 305 genes remained after exclusion of potential copy number variations (CNVs). These focal aberrations were significantly enriched for cancer census genes. Expression analysis of genes residing within the most frequently occurring focal aberrations revealed concurrent altered expression in CIN2/3 and/or cervical carcinomas compared to normal cervical samples for ATP13A3, HES1, OPA1, HRASLS, EYA2, ZMYND8, APOBEC2, NCR2 and hsa-miR-375.

EYA2 and hsa-miR-375, located within a top focal gain (20q) and loss (2q), respectively, were chosen for further functional validation. Gene silencing of EYA2 in HPV16-transformed keratinocytes significantly reduced viability, migratory capacity and anchorage-independent growth of these cells. For hsa-miR-375 a direct correlation between a (focal) loss and significantly reduced expression was found. Ectopic expression of hsa-miR-375 in SiHa and CaSki cells reduced cellular viability.

These data provide a proof of concept that chromosomal aberrations can contribute to HPV-induced carcinogenesis and has led to the identification of EYA2 and hsa-miR-375 as oncogene and tumour suppressor gene, respectively. Consequently, functional analysis of the remaining candidate driver genes identified in the top focal aberrations is warranted. Finally, the potential value of these driver genes as either disease marker or therapeutic target needs to be determined.

Silencing of MAL by DNA methylation functionally contributes to HPV-mediated transformation

In Chapter 5 candidate disease marker MAL, identified by previous microarray expression analysis of cervical carcinomas\textsuperscript{10}, was studied. The mechanism
underlying MAL silencing, DNA methylation status and its functional role in cervical carcinogenesis was investigated using cell lines, cervical biopsies and cytological samples. MAL mRNA expression was shown to be severely downregulated in HPV-immortalised cell lines and cancer cell lines, which could be reversed upon treatment with a demethylating agent. The epigenetic regulation of MAL expression was confirmed by the detection of DNA methylation at two promoter regions using quantitative methylation-specific PCR (qMSP) in HPV-immortalised cell lines and cervical cancer cells. A functional role for methylation-mediated silencing of MAL was demonstrated in SiHa cervical cancer cells, in which ectopic expression of MAL suppressed proliferation, migration and anchorage-independent growth. Furthermore, the frequency of promoter methylation at both promoter regions increased with the severity of cervical lesion grade: 0% in normal tissue, 9% in CIN1, 53% in CIN3, 90% in SCC and 93% in AdCa. MAL methylation could also be detected in cervical scrapes and was found to be significantly increased in women with underlying CIN2/3 compared to controls. Moreover, MAL promoter methylation was significantly correlated to reduced mRNA expression in both cervical biopsies and scrapes. These data indicate that testing for both reduced MAL mRNA expression and increased promoter methylation may be used for the risk-stratification of hrHPV-positive women.

Subsequent analysis of a large series of cervical tissue specimens and prospectively collected hrHPV-positive scrapes, showed that the detection of CIN3+ by MAL promoter methylation can be improved by additive testing for promoter methylation of CADM1, another gene found to be functionally involved in HPV-mediated transformation. A recent study on hrHPV-positive scrapes derived from a large population-based screening study (POBASCAM) revealed that combined testing for MAL and CADM1 promoter methylation was at least equally discriminatory for CIN3+ as cytology, either or not in combination with HPV16/18 genotyping.

**CADM1 and MAL DNA methylation levels in hrHPV-positive cervical scrapes increase proportional to severity of CIN disease, duration of preceding hrHPV infection and cancer**

In Chapter 6, levels of CADM1 and MAL promoter methylation were examined in a screening cohort of hrHPV-positive women and cancer patients using quantitative methylation-specific PCR. These included hrHPV-positive cervical scrapes of women with no underlying disease in their follow-up (≤CIN1, n=167; of which 140 with normal cytology) and women with CIN2/3 (n=54; of which 19 with normal cytology). Additionally, methylation levels were examined in hrHPV-positive cervical scrapes of women with CIN2/3 following a short-term (<5 year, n=19) and long-term (≥5 year, n=29) PHI, respectively, a surrogate of lesion age (Chapter 3), as well as women with carcinoma (n=44). Next to histology (≤CIN1, CIN2/3 and cancer), CIN2/3 was categorised according to increasing severity of the underlying cervical lesion based on < and ≥5yrPHI, which were assigned a respectively lower and higher disease stage. In scrapes with an unknown duration of PHI cytological classification was included as an alternative proxy for disease staging. hrHPV-
positive cytologically normal scrapes of women with ≤CIN1 were taken as a reference.

Methylation levels of both genes were significantly higher in scrapes of women with underlying CIN2/3 compared to ≤CIN1-normal cytology (the reference group). Overall methylation levels in CIN2/3 were increased 5.3- and 6.2-fold for CADM1 and MAL, respectively, compared to the reference group. The increase in methylation levels for CADM1 and MAL in CIN2/3<5yrPHI were 3.0- and 3.6-fold compared to the reference group, respectively. CIN2/3≥5yrPHI had further elevated methylation levels, being 11.5- and 13.6-fold increased compared to the reference group. The increase in methylation levels in CIN2/3≥5yrPHI compared to CIN2/3<5yrPHI was significant. Women with cervical carcinoma displayed the highest methylation levels, i.e. 143.5- and 453.9-fold increase compared to the reference group, which was significantly higher compared to both CIN2/3<5yrPHI and CIN2/3≥5yrPHI.

The increase in methylation levels with disease progression, even within the CIN2/3 group, could be validated upon taking cytology as an alternative proxy for disease staging. Per histological class, methylation levels were lower for samples with normal cytology compared to abnormal cytology. Interestingly, methylation levels in women with CIN2/3-normal cytology and ≤CIN1-abnormal cytology were comparable to those detected in women with CIN2/3<5yrPHI. This is in agreement with low to intermediate levels of methylation being indicative of a relatively new CIN2/3, or a potentially regressive lesion, and does not point to a high short-term progression risk for carcinoma. In fact, methylation levels were higher in more advanced CIN2/3 (≥5yrPHI) and dramatically increased in case of cervical cancer, indicating that cancers are unlikely to be missed by these methylation markers. Based on present findings it may be suggested that undetectable or very low methylation levels point to a low or negligible risk of cervical disease. Intermediate levels indicate potential risk of underlying disease, warranting close surveillance. High methylation levels indicate the presence of clinically relevant disease requiring immediate referral to the gynaecologist.

The main findings described in this thesis are summarised in Figure 1 and placed into sequential order.

**Future perspectives**

*Pathogenesis and disease marker discovery*

In this thesis it was shown that the genetic and epigenetic heterogeneity of CIN2/3 is at least in part dependent on the duration of preceding hrHPV infection. Moreover, the extent of chromosomal aberrations is also related to the hrHPV type present. As the majority of CIN2/3 with a relatively short (<5 years) preceding infection hardly showed any chromosomal aberrations, one could speculate that these lesions are still at a very low risk for progression. Upon viral persistence
Figure 1: Schematic overview of host cell aberrations during cervical cancer development. Gain of 3q is a prominent event during carcinogenesis, witnessed with highest frequency in lesions with HPV16. Other high-risk HPV types seem to need many additional chromosomal aberrations, with main events being gain of chromosome 1 and loss on chromosome 2q. Downregulation, for instance via promoter methylation, of tumour suppressor MAL occurs relatively early in high-grade lesions, followed by inactivation of CADM1. Downregulation of hsa-miR-375, located at 2q, via copy number loss has been observed in high-grade lesions, though (additional) downregulation may be due to promoter methylation. The copy number gain of 20q is correlated to the upregulation of the for cervical cancer newly defined oncogene EYA2. Of note the extent and the specific manner in which genes may be affected during progression of cervical lesions may well depend on the particular hrHPV type a woman is infected with.

(≥5 year infection) the number of aberrations and regions affected appear, in part, related to the hrHPV type present. HPV16-positive lesions displayed only few chromosomal aberrations with gain of 3q being most prominent. Direct comparison of HPV16- to HPV31-positive lesions revealed specific regions to be significantly more affected in lesions with HPV31, pointing to HPV type dependent molecular pathways of cervical carcinogenesis. To further substantiate the finding that chromosomal profiles are at least in part HPV type dependent, an extension of the sample series with other hrHPV types, as well as with invasive carcinomas, is warranted. Additionally, a recently developed in vitro model system of keratinocytes transformed by different hrHPV types may be of value to further
investigate potential HPV type specific chromosomal aberrations and pathways of transformation.

Epidemiological studies have indicated different risks of progression towards carcinoma for specific HPV types, which may in part be linked to the molecular differences described in this thesis. While HPV16 and 18 represent the majority of cervical cancer cases, other types are less frequently found in carcinomas and the age of the women diagnosed is higher compared to those infected with one of the frequent types.\textsuperscript{27} That also the clinical behaviour of high-grade CIN is heterogeneous can be concluded from data described by McCredie \textit{et al.}\textsuperscript{4} In an unethical study in New Zealand the progression rate of CIN3 was analysed of women who had been withheld treatment. From these data it was deduced that approximately 50\% of CIN3 will not progress towards carcinoma (within a time span of 30 years) if left untreated.

To determine the functional relevance of the observed chromosomal changes to the transformation process of the HPV-infected cell, the driver gene(s) located within these chromosomal aberrations need(s) to be identified. Whereas this is quite difficult for the large chromosomal aberrations commonly observed in cervical (pre)cancer, the additional identification of small, focal aberrations can greatly improve the identification of such potential driver genes. This was shown for EYA2 and hsa-miR-375, located in a focal gain and loss on 20q and 2q, respectively, in Chapter 4. Interestingly, alternative silencing of hsa-miR-375 via promoter methylation has been described in melanoma and gastric cancer.\textsuperscript{28,29} Due to the limited amount of material from the microdissected lesions, the methylation status of hsa-miR-375 could not be examined to assess the correlation between (focal) loss, reduced gene expression and promoter methylation. Preliminary data indicate that methylation of hsa-miR-375 is also apparent in cervical carcinomas (Wilting \textit{et al}, unpublished results). The promising results obtained for both EYA2 and hsa-miR-375 warrant further analysis of the other identified genes located in focal aberrations as listed in Table 1 of Chapter 4. Together, these genes provide potential candidate biomarkers to be applied as triage markers in cervical screening or may even serve as targets for therapeutic intervention upon proof of functional involvement in HPV-mediated transformation.

Interestingly, analysis of the arrayCGH profiles for focal aberrations indicated the presence of intragenic copy number breakpoints for several genes located at the borders of these aberrations. Such breakpoints may indicate viral integration sites or chromosomal rearrangements. It is possible that fusion genes are formed, of which the gene products may act as functional oncproteins.\textsuperscript{30} In our profiling studies the Agilent 105K arrayCGH platform was used for the detection of chromosomal aberrations, which still has a limited resolution compared to the 1M (1 million probes) arrays currently available. As massive parallel sequencing (MPS) is now becoming affordable, this will likely be the method of choice for further analysis of both clinical specimens and different stages of \textit{in vitro} HPV-immortalised cells representing different hrHPV types in the near future. MPS will provide even
more information on the genetic environment of a (pre)cancer as, in addition to copy number aberrations, also the presence of insertions/deletions, chromosomal rearrangements, point mutations and fusion genes can be assessed in a single experiment. Implementation of MPS and integration with genome-wide DNA promoter methylation and/or mRNA and miRNA expression analysis will further elucidate the complexity of events underlying cervical carcinogenesis and will aid to narrow down suitable candidate markers. Using such an integrative approach, more insight will be gained into the different or additional mechanisms employed by various HPV types to induce malignant transformation.

Though beyond the scope of this thesis, suitable marker candidates capable of predicting malignant progression, or to be used as treatment target, may also be based on changes within the virus itself. For instance, the presence of integrated viral copies could be used to estimate underlying cancer risk, as integration has been linked to progression and may contribute to deregulated expression of E6 and E7 through loss of the repressive function of E2. Interestingly, the integration frequency in CIN3 and cancers was found to be HPV type dependent, indicating that the manner of E6E7 deregulation may also be type-dependent. Another factor contributing to deregulated E6/E7 expression is methylation-mediated silencing of the HPV E2 binding sites in the viral long control region (LCR), which also has been proposed to be a marker for progression of cervical disease. Based on the type-dependent differences in chromosomal aberrations as described in this thesis, as well as type-dependent integration frequencies, it may be speculated that methylation-mediated silencing of E2 is also related to HPV type. Hence, upon analysis of markers based on both host cell and viral alterations the specific HPV type present needs to be taken into account.

Biomarker validation and clinical applications

In the near future, HPV-testing is likely to become the primary cervical cancer screening tool in the Netherlands. Though triage of women participating in the regular screening programme may firstly be based on cytological examination, this test is not optimal and in case of self-collected cervico-vaginal samples no such examination can be performed. Therefore it is imperative to have objective biomarkers to aid risk-stratification of hrHPV-positive women. The research results described in this thesis enable a more objective selection of CIN2/3 with a high risk of progression for use in biomarker validation studies. Based on the HPV type present combined with the specific chromosomal profiles a prediction can be made of the short-term progression risk of the lesions and as such their need of detection by triage markers. Such markers should be capable of indicating risk independent of hrHPV type, or should be complementary to take various hrHPV type-related mechanisms into account.

Biomarkers for triage of hrHPV-positive women could be based on the chromosomal aberrations detected in CIN2/3 with long-term hrHPV infection (Chapter 2 and 3) as well as the specific focal aberrations described in Chapter 4. Specific copy number aberrations can be detected in cervical scrapes with
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fluorescence *in situ* hybridisation (FISH), qPCR or multiplex ligation-dependent probe amplification (MLPA). For example, FISH for TERC (3q26) has been performed on cervical scrapes, revealing a majority of the samples underlying CIN2/3 to show copy number gain.\textsuperscript{22,34} An MPS-based approach in which a select number of aberrations can be tested in a high-throughput fashion, using for instance the MiSeq (Illumina), may for example become the preferred method of choice. Yet the ultimate selection depends on the sensitivity of the particular technique to detect the (pre)cancer biomarker and the amount of input material required and available. Ideally, a technique with a low detection limit and a high reproducibility requiring only a minimal amount of input material would be the optimal choice.

Although DNA methylation analysis shows great promise as triage test for hrHPV-positive women on scrapes and cervico-vaginal lavages (Hesselink *et al*, in preparation)\textsuperscript{26,35} it has yet to be determined whether lesions missed by these novel molecular markers reflect those in need of immediate treatment. Based on the findings described in Chapter 6 it could be argued that low/no methylation levels indicate a low short-term progression risk. A systematic analysis of lesions negative for the current methylation markers for other/additive chromosomal or epigenetic (including miRNA) aberrations will reveal if the current marker panel can be expanded and improved upon, or whether these lesions are not yet affected at the molecular level and may therefore still have a very low progression risk. These studies are currently in progress.
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