Chapter 1

General Introduction
Cervical cancer

Epidemiology

With an estimated occurrence of 530,000 new cases and approximately 275,000 deaths in 2008, cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females worldwide. The age standardised incidence rate (ASIR) and age standardised mortality rate (ASMR) of cervical cancer in developed countries (USA and Western Europe) is 9 and 3 per 100,000, respectively. The incidence of cervical cancer is highest in developing countries without population-based screening programmes, where 86% of the cases and 88% of related deaths occur. In these countries, the ASIR and ASMR is 18 and 10 per 100,000, respectively (see Figure 1).

![Figure 1: World-wide age standardised incidence rate (ASIR) of cervical cancer 2008. Adapted from Arbyn et al.](image)

In 2008, the ASIR in the Netherlands was 6.8 per 100,000 and the ASMR 1.9 per 100,000. These rates are a reflection of the implementation of the screening program, which was introduced in the mid 70s and has lead to a reduction of both incidence and mortality.

The uterine cervix consists of two parts: the endocervix (inner part of the cervix on the uterine side), which is covered by columnar epithelium, and the ectocervix
(outer part of the cervix on the vaginal side), which is lined by non-keratinising squamous epithelium. The boundary between these two types of epithelium is called the squamo-columnar junction (SCJ). Due to hormonal changes occurring from puberty onwards, the original SCJ present at time of birth will shift inwards, whereby the columnar epithelium of the endocervix is replaced by squamous epithelium of the ectocervix. The area between the old and new SCJ is called the transformation zone. This metaplastic squamous epithelium is assumedly most vulnerable to oncogenic transformation. There are different histotypes of cervical cancer, with the main types being squamous cell carcinoma (SCC), adenocarcinoma (AdCa) and adenosquamous carcinoma.

**Human papillomavirus infection**

Cervical cancer is a rare complication of a persistent infection with certain human papillomavirus (HPV) types. HPVs are double-stranded DNA viruses belonging to the papillomaviridae family, of which there are more than 130 different types. HPVs can be grouped into genera (α, β, γ, µ, η), with types in the same genus sharing common characteristics, such as tissue tropism and oncogenic potential (Figure 2).³ A subset of HPV types from the α-genus can infect the lower anogenital tract. Depending on the oncogenic potential, HPV types can be classified as low-risk or high-risk. Anogenital infection with low-risk types is mainly associated with benign warts.⁴ Persistent infection with high-risk HPV (hrHPV), on the other hand, has been causally related to the development of cervical cancer and the virus can be detected in virtually all carcinomas.⁵,⁶ These hrHPV types belong to the α5, α6, α7, α9 and α11 species. Approximately 13 hrHPV types constitute the most important types causing cervical cancer.⁷ HPV types 16 (α9) and 18 (α7) are the cause of approximately 70% of all cervical cancers.⁸
Figure 2: Phylogenetic tree of HPV.
The genome of HPV is ~8 kb and contains eight viral genes and a long control region (LCR). The genes are grouped into early genes (E1, E2, E4, E5, E6, E7), expression of which is necessary for viral replication, and late genes (L1, L2), which encode the major and minor capsid proteins (Figure 3). The functions of the viral proteins are summarised in Table 1. In productive infections the expression status of these genes is dependent on the stage of the HPV life cycle.

![Figure 3: Schematic representation of the genomic structure of HPV, HPV16 in particular. Late genes: L1 and L2; Early genes: E1, E2, E4, E5, E6, E7; LCR: Long Control Region (adapted from Stanley et al)].

A productive infection begins when viral particles gain access to the epithelial basement membrane, most likely via micro-abrasions. From there these particles are able to infect the basal cells, though the exact manner has not yet been fully cleared up. Initial binding and/or uptake of the virus is suggested to occur via heparin sulphate proteoglycans and is mediated by secondary receptors for efficient infection, perhaps by the α6-integrins. In infected cells the viral genome is replicated with the cellular DNA during S-phase and maintained as stable episomes in these cells. Expression of the viral proteins occurs at extremely low levels, which facilitates escape from the immune system. As the basal cells undergo cell division, one of the daughter cells is pushed up to the supra-basal compartment. This is accompanied by a differentiation process, during which these cells exit the cell cycle. Upon differentiation, viral differentiation-dependent promoters become upregulated, resulting in an increased expression of
viral genes. In the upper layer of the cervical epithelium, the virus undergoes the last stage of its life cycle and new viral particles are packaged and shed when the cells reach the epithelial surface.

In case of a transforming infection the normal viral life cycle is aborted. Such infections are characterised by overexpression of the E6 and E7 oncogenes in the proliferating basal cells of the epithelium. The exact mechanism contributing to deregulated expression of E6/E7 is not quite understood: one possible manner may be via viral integration into the host cell genome, in which case the repressive function of E2 on E6 and E7 transcription is lost.\(^{20}\) However, not all carcinomas contain integrated viral copies and deregulated E6/E7 expression may occur prior to integration, for example via methylation-mediated silencing of the HPV E2 binding sites.\(^{21}\) In addition, recent data seem to indicate that integration is a consequence of increasing genomic instability rather than the cause.\(^{22}\)

The E6 and E7 proteins have multiple interaction partners (reviewed by Korzeniewski et al\(^{23}\)) of which the interactions with tumour suppressors p53 and pRb, respectively, are characterised best. E6 prevents cells from undergoing p53-mediated apoptosis upon DNA damage by mediating ubiquitination and degradation of p53.\(^{24}\) By binding to pRb family members E7 mediates loss of their function, resulting in transactivation of cellular proteins required for (viral) DNA replication via E2F. The loss of cell-division control due to combined overexpression of E6 and E7 in proliferating cells will eventually lead to genomic instability and the accumulation of gene mutations that affect oncogenes and tumour suppressor genes, which may be the driving force for progression to invasive carcinoma.\(^{8,25}\)

The release of the negative feedback loop of pRB on E2F also results in increased expression of the tumour suppressor p16\(^{\text{INK4a}}\). In addition, independent of pRb-binding, E7 expression in proliferating cells induces the transcriptional activity of histone demethylase KDM6B, resulting in p16\(^{\text{INK4a}}\) upregulation.\(^{26}\) Thus, overexpression of p16\(^{\text{INK4a}}\) can be considered a marker for transforming hrHPV infections.\(^{27}\)

**Table 1:** The main functions of the viral proteins.\(^{23}\)

<table>
<thead>
<tr>
<th>HPV protein</th>
<th>Function</th>
<th>Temporal expression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E1</strong></td>
<td>Viral replication, DNA helicase</td>
<td>Early (prior to productive viral replication)</td>
</tr>
<tr>
<td><strong>E2</strong></td>
<td>Viral replication, origin binding, transcriptional activation and repression, tethering of viral DNA to host chromosomes</td>
<td>Early</td>
</tr>
<tr>
<td><strong>E4</strong></td>
<td>Destabilisation of cytokeratin network</td>
<td>Early and late</td>
</tr>
<tr>
<td><strong>E5</strong></td>
<td>Mediates mitogenic signaling of growth factors</td>
<td>Early and late</td>
</tr>
<tr>
<td><strong>E6</strong></td>
<td>Major oncoprotein</td>
<td>Early</td>
</tr>
<tr>
<td><strong>E7</strong></td>
<td>Major oncoprotein</td>
<td>Early</td>
</tr>
<tr>
<td><strong>L1</strong></td>
<td>Major viral capsid protein</td>
<td>Late (at the time of productive viral replication)</td>
</tr>
<tr>
<td><strong>L2</strong></td>
<td>Minor viral capsid protein</td>
<td>Late</td>
</tr>
</tbody>
</table>
Progression to cervical carcinoma

While infection with hrHPV is common, more than 80% of the hrHPV infections are cleared by the immune system. Only in some instances precancerous lesions arise, the so-called cervical intraepithelial neoplasia (CIN) in case of SCC. Histologically, these lesions can be divided into low-grade (CIN1, mild dysplasia) and high-grade (CIN2/3, moderate/severe dysplasia) lesions, depending on the severity of the disturbance of the cervical epithelium (Figure 4). Not all CIN represent true precursor stages of cervical cancer. Low-grade CIN are associated with productive viral infections of either low-risk or high-risk HPV types. High-grade CIN with the potential of progression to invasive carcinomas are associated with transforming hrHPV infections.4

Figure 4: Schematic representation of the consecutive histological stages of cervical disease (adapted from Lowy et al28). Shown are the stages for Cervical Intraepithelial Neoplasia (CIN) and associated squamous cell carcinoma (SCC).

High-grade CIN may be rapidly induced within 2-3 years following hrHPV infection (Figure 5).25,29 Their development may in part be related to the hrHPV type a woman is infected with, as hrHPV-positive women with normal cytology have different risks of high-grade CIN depending on hrHPV type.30 Not all high-grade CIN will progress to carcinoma31 and various studies have reported spontaneous regression of high-grade CIN in 19-50% of the cases in a time period of 13 weeks up to 2 years.32-36 Progression of high-grade CIN to carcinoma also appears to be dependent on the hrHPV type present, since the prevalence of specific hrHPV types is different between high-grade CIN and SCC.37-45 This is likely a reflection of the different oncogenic potential of different hrHPV types combined with the various success rates of viral persistence and evasion of the immune system. Progression of persistent high-grade CIN towards invasive carcinoma may take an additional 10-30 years (Figure 5).31
Cervical cancer screening in the Netherlands

Due to the long interval required to develop invasive carcinoma, cervical cancer is considered a preventable disease. Introduction of population-based screening programmes has resulted in a decreased incidence.\textsuperscript{46-48} The current screening programmes are based on cytological assessment of cervical scrapes, the Papanicolaou (Pap) test. With this test, dysplastic cells derived from the (pre)malignant disease stages can be detected. The Pap classification has been improved in recent years, resulting in the Bethesda 2001 classification currently used in most countries.\textsuperscript{49} In the Netherlands, cervical scrapes are assessed according to the CISOE-A classification system (Composition, Inflammation, Squamous epithelium, Other abnormalities and endometrium and Endocervical columnar epithelium - Adequacy of the smear), as specified in Table 2. However, cytology is only an indication of underlying histological abnormalities and colposcopy-directed biopsies are necessary to confirm the presence of clinically relevant lesions. Cytological screening is not optimal and the sensitivity of this test for the detection of relevant disease (CIN2+) is approximately 65%; thus, a relatively high proportion of relevant cases are still missed using this test.\textsuperscript{50-52} Furthermore, cytological screening has a subjective nature, reflected by a relatively high proportion of false-positive cases and high inter- and intra-observer variations in cytological classification of abnormal smears.\textsuperscript{52,53}

The witnessed decrease in the incidence of cervical carcinoma upon introduction of the screening programme can mainly be attributed to the early detection of premalignant lesions preceding SCC (accounting for \textasciitilde80\% of cervical cancer cases). For the histological subtype AdCa (accounting for 10-20\% of cases) the incidence has remained similar or even slightly increased in developed
This most likely reflects the fact that (precursors of) AdCa are situated higher up in the endocervix and are more difficult to detect with cytology.\textsuperscript{46,54,55} Moreover, reliable cytological criteria for AdCa precursors are presently lacking.

**Table 2:** CISOE-A classification used in the Netherlands versus the Pap and Bethesda classification (adapted from Bulk \textit{et al}\textsuperscript{56}).

<table>
<thead>
<tr>
<th>Description</th>
<th>S</th>
<th>O</th>
<th>E</th>
<th>Bethesda 2001</th>
</tr>
</thead>
<tbody>
<tr>
<td>inadequate scrape</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>unsatisfactory for evaluation</td>
</tr>
<tr>
<td>normal</td>
<td>1</td>
<td>1</td>
<td>1-2</td>
<td>NILM</td>
</tr>
<tr>
<td>normal</td>
<td>1</td>
<td>2</td>
<td>1-2</td>
<td>atrophy, NILM</td>
</tr>
<tr>
<td>borderline dyskaryosis</td>
<td>2-3</td>
<td>3</td>
<td>3</td>
<td>ASCUS/ASCH</td>
</tr>
<tr>
<td>mild dyskaryosis</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>ASCH/LSIL</td>
</tr>
<tr>
<td>moderate dyskaryosis</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>HSIL</td>
</tr>
<tr>
<td>severe dyskaryosis</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>HSIL</td>
</tr>
<tr>
<td>carcinoma \textit{in situ}</td>
<td>7</td>
<td>-</td>
<td>7</td>
<td>HSIL, AGC, favour neoplastic</td>
</tr>
</tbody>
</table>

*Abbreviations: CISOE-A, Composition, Inflammation, Squamous epithelium, Other abnormalities and endometrium and Endocervical columnar epithelium - Adequacy of the smear; LSIL, Low-grade Squamous Intraepithelial Lesion, HSIL, High-grade Squamous Intraepithelial Lesion; NILM, Negative for Intraepithelial Lesion or Malignancy; ASCUS, Atypical Squamous Cells of Undetermined Significance; ASCH, Atypical Squamous Cells cannot exclude HSIL; SCC, Squamous Cell Carcinoma; AGC, Atypical Glandular Cells; AIS, Adenocarcinoma \textit{In Situ}; AdCa, Adenocarcinoma.*

The Netherlands has a population-based screening system with call and recall. Since 1996, women between 30 and 60 years old are screened. Women with normal cytology are recalled after 5 years, women with borderline or mild dyskaryosis (ASCUS/LSIL) are advised to repeat the test after 6 and 18 months and in case of moderate dyskaryosis or worse (HSIL) women are directly referred to colposcopy.

Since hrHPV is nearly always detectable in cervical cancer and its high-grade precursors, testing for hrHPV in routine screening may prove to be of value. In Sweden, Italy, the United Kingdom, Finland, Canada and the Netherlands randomised controlled trials have been initiated to establish the performance of hrHPV-testing as a primary cervical screening tool.\textsuperscript{57-63} These include the Dutch POBASCAM trial (44,102 women) and VUSA-screen study (50,000 women).\textsuperscript{57,64,65}
**General Introduction**

**Cervical screening trials in the Netherlands**

The POBASCAM trial was initiated to evaluate the effectiveness of hrHPV-testing in conjunction with cytology (intervention group) to that of classical cytological screening (control group). This large randomised trial was embedded in the regular population-based screening programme. Information of women participating in this trial is available from two consecutive screening rounds (10 years). In this trial hrHPV-testing was performed by GP5+/6+ PCR followed by an enzyme immunoassay (EIA). The GP5+/6+ PCR targets the L1 gene of the viral capsid and is capable of detecting both low- and high-risk HPV types. The read-out involves an EIA using probe cocktails representing low-risk and/or high-risk HPV types (i.e. hrHPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68).

The VUSA-screen study was an intervention study embedded in the regular population-based screening programme, designed to evaluate the effectiveness of combined cervical cytology screening with HPV-testing by use of the hybrid capture 2 (HC2) test. The HC2 test is a nucleic acid hybridisation assay where specimens containing the target DNA hybridise with a specific HPV RNA probe mixture capable of detecting 13 hrHPV types (same as GP5+/6+, but without type 66). The VUSA-screen study had two aims: (1) to evaluate the effectiveness of hrHPV triage in women with borderline of mild dyskaryosis (BMD) by comparing current screening protocols using conventional cytology with a strategy where women with BMD were advised according to their hrHPV test result; (2) to evaluate the risk for developing high-grade CIN in cytological normal women with an hrHPV-positive versus hrHPV-negative test result. This study has follow-up data available for up to 5 years (median 3 years).

The results of these studies indicated hrHPV-testing to be more sensitive than cytological assessment of cervical scrapes for the detection of CIN2+. However, as with hrHPV-testing also a substantial number of transient infections are detected, screening by hrHPV-testing only would lead to increased colposcopy referral rates, overdiagnosis, overtreatment and increase of costs as compared to sole cytological screening. In order to reduce the number of colposcopies of hrHPV-positive women, guidelines for clinical validation of a given hrHPV test have been developed. In addition, further stratification by means of triage testing for hrHPV-positive women is recommended. Based on data of the VUSA-screen, 14 triage strategies for hrHPV-positive women were examined. In the Netherlands, where a high quality cytological screening programme is present as substantiated by a high sensitivity for CIN2+, a low rate of cytological abnormalities (high specificity) and a long screening interval (5 years), the most feasible management strategy that yielded a high negative-predictive value and a modest colposcopy referral rate for CIN3+ appeared to be initial hrHPV-testing with cytology triage, followed by repeat cytology testing after 6 months - 1 year for those women who have hrHPV-positive, cytological normal scrapes at baseline. It is of note that in countries with lower quality of cytology and higher rates of cytological abnormalities, shorter screening intervals and higher colposcopy rates, other triage strategies may be
considered (for instance combined cytology and HPV-genotyping at baseline for types 16/18/31/33/45 without repeat testing). This is dependent on the positive-predictive value for colposcopy referral for CIN2+, accepted by the health authorities and gynaecologists, and may differ from country to country.

In May 2011 the Dutch Health Council advised the Minister of Health to implement hrHPV-testing as the primary screening tool. For hrHPV-negative women of 40 years or older, the recall period can be extended, resulting in less visits to the general practitioner. In case of hrHPV-positivity, cytological samples will be examined for abnormal cells. Women with evidence of abnormal cells will be referred to the gynaecologist while hrHPV-positive women with normal cytology will be subjected to more close surveillance.

**HPV-testing on self-collected cervico-vaginal specimens**

An added value of hrHPV-testing is that it also can be applied effectively on self-collected cervico-vaginal samples. The majority of the cervical cancer cases (approximately 50%) are diagnosed in non-responders, i.e. women that do not participate in the screening programme. Non-responders may be sent self-samplers, which they themselves can use to collect cervico-vaginal cells. These samples are subsequently sent to the laboratory for evaluation. A recent study by Gök et al has demonstrated that offering self-sampling to these non-responder women can engage a substantial proportion of them into the screening program. However, in self-samples only the presence of HPV DNA can be determined, as cytological examination has a low sensitivity due to the fact that these self-obtained samples mostly contain vaginal cells and only a few intact abnormal cervical indicator cells. Therefore, hrHPV-positive women would still need to visit their general practitioner to have a cervical smear taken in order to determine the presence of abnormal cells.

**Heterogeneity of high-grade CIN**

With the hrHPV-test soon becoming the primary screening tool it is imperative to have additional biomarkers for risk-assessment of hrHPV-positive women. Ideally, an objective read-out should be available that is applicable to self-sampled specimens as well. These biomarkers may be based on host cell aberrations. For example, promoter methylation of tumour suppressor genes, increased mRNA expression of oncogenes or onco-miRs, or the presence of specific chromosomal aberrations underlying the HPV-mediated transformation. Due to the long interval between the establishment of a high-grade CIN to progression to invasive carcinoma, the short-term progression risk of high-grade CIN is highly variable, which may confound results of biomarker studies for triage purposes. Even in histological, morphologically similar lesions that are p16\textsuperscript{INK4a}-immunopositive, current tests cannot distinguish high-grade CIN that have been present for a relatively short time from those present for a much longer time, which are therefore likely more advanced and have a higher short-term risk of progression.
towards carcinoma. Lesions that have been present for a long time have had more time to accumulate aberrations compared to lesions that have only recently been established. As a consequence, biomarker results may vary between different studies depending on the proportion of ‘incident’ versus ‘prevalent’ high-grade CIN. Distinguishing these lesions might be important since only prevalent high-grade CIN have a short-term progression risk. As high-grade CIN are treated upon detection, it was up till now not possible to determine the duration of existence of these lesions. With aid of the longitudinal studies using hrHPV-testing as screening tool, information is now available over two consecutive screening rounds, enabling an estimation of the duration of existence of premalignant lesions. Furthermore, heterogeneity with respect to HPV type related aspects may also be assessed.

Once a beginning has been made to elucidate the heterogeneity of high-grade CIN, suitable screening biomarkers may be selected to aid the screening programme in an objective manner. Therefore it is necessary to first obtain insight into (epi)genetic aberrations that underlie the development of CIN2+.

**Host cell aberrations associated with cervical carcinogenesis**

Infection with hrHPV and expression of the viral oncogenes in dividing cells is the first step in the development of cervical carcinoma, yet additional events within the host cell genome are necessary to drive malignant progression. These include both genetic and epigenetic events that result in the functional abrogation of tumour suppressor genes or the activation of oncogenes and may be achieved by various mechanisms. Alterations that have been described in the context of carcinogenesis include mutations, methylation of gene promoter regions, allelic loss (loss of heterozygosity) and/or chromosomal aberrations. In the following sections these events will be described primarily for the histological subtype SCC and its precursor CIN.

**Mutations**

To date, relatively few mutations in (candidate) tumour suppressor genes and oncogenes have been described for cervical cancer. Tumour suppressors p53 and pRb, frequently mutated in human cancers, are inactivated by the viral oncogenes E6 and E7 and only rarely mutated in cervical cancer (6% and 3%, respectively). Other genes with reported mutations in cervical carcinoma (in studies with >100 samples) are inactivating mutations in tumour suppressor genes STK11 (13%), CDKN2A (9%) and PTEN (5%) and activating mutations in oncogenes PIK3CA (11%), HRAS (9%), KRAS (8%), FGFR3 (2%), NRAS (2%) and BRAF (1%). Mutation frequencies of these genes in CIN have either not been studied at all, or only in studies with small sample series. The currently available data show either no or only a very low frequency of respective mutations in high-grade CIN. It is possible that more DNA mutations will be detected using next generation sequencing techniques.
**Epigenetic silencing of tumour suppressor genes**

DNA methylation can be detected both in cervical tissues as well as in cervical scrapes. One of the techniques used to detect methylation is (quantitative) methylation-specific PCR (MSP). This technique is based on the conversion of unmethylated cytosine to uracil by bisulphite treatment of the DNA, whereby methylated cytosines preceding guanine (CpG) are protected from conversion. Following conversion, (real-time) PCR using primers specific for the methylated (i.e. not converted) sequence is performed.

Aberrant methylation patterns have been described for a diverse number of candidate tumour suppressor genes in cervical (pre)cancers (reviewed by Wentzensen *et al*). Genes that have been examined in more than 5 studies are DAPK1, RASSF1, CDH1, CDKN2A, MGMT, RARB, APC, FHIT, MLH1, TIMP3, GSTP1, CADM1, CDH13, HIC1 and TERT. The most frequently methylated genes, based on weighted mean frequencies by study size, in cervical cancer (SCC and AdCa combined) are CDH1 (58%), DAPK1 (57%), CADM1 (55%) and TERT (55%). Methylation frequencies for some of these genes varied widely across studies (TIMP3, MGMT and CDH1), whereas other genes were either rarely or not methylated in cervical cancer (RASSF1, GSTP1 and MLH1). Since DNA methylation of certain genes can be detected in precancerous lesions, methylation-based biomarkers may provide a suitable tool to improve cervical screening algorithms. DNA methylation in high-grade CIN or its cytological equivalent of the most examined genes mentioned above, revealed the weighted mean methylation frequencies to be highest for CADM1 (33%), followed by CDH1 (29%), DAPK1 (29%) and TERT (29%).

**Loss of heterozygosity (LOH)**

Allelic loss, or loss of heterozygosity, seems more prevalent in cervical disease. Frequently affected regions in carcinomas are reported for chromosomes 3p, 4, 5, 6p, 8p, 10q, 11, 17p, 18q and 19q. CIN may also be affected by LOH, for instance at regions 3p, 6p and 11q, which are frequently affected in carcinoma as well. Loss of tumour suppressor genes within these regions thus seems an important event that occurs early during carcinogenic progression. Genes residing within affected regions are for example FHIT (3p) and TNF-α (6p), both of which have been implicated in carcinogenesis.

**Chromosomal aberrations underlying development of high-grade CIN**

A commonly used technique for the detection of chromosomal aberrations on a genome wide level is comparative genomic hybridisation microarray (arrayCGH). DNA from cervical (pre)cancerous tissue (sample) may be compared to that of normal DNA (reference) by labeling the sample and reference DNA with different fluorescent dyes, followed by hybridisation to an array. This array contains oligonucleotides (the number depending on the platform used) representing sequences spanning the entire genome. Both sample and reference will be able to hybridise to the array. The resulting fluorescent signal for each specific oligonucleotide then provides information on the presence of extra (gain) or less
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(loss) chromosomal copies for that locus in the sample.

Gains of chromosomal regions may result in the increased expression of genes (oncogenes) within the affected regions, whereas lost chromosomal regions may in turn result in decreased expression (tumour suppressor genes). In previous studies both cervical lesions and carcinomas have been examined for their chromosomal aberrations. However, due to relatively low resolution of the used techniques the reported aberrations encompass multiple megabases, this impedes the identification of genes that are directly responsible for tumour development (driver genes). With the advent of new and higher resolution platforms to determine chromosomal aberrations, it is now possible to narrow down the size of affected regions. The most frequent events in SCC are gains at chromosomes 1q, 3q and 20q and losses at 2q, 3p, 4q, 6q, 11q and 13q. Chromosomal aberrations have also been investigated in premalignant stages (literature data are summarised for high-grade CIN in Table 3). Aberrations found in these lesions include the gains and losses present in SCC. However, the frequencies with which aberrations are detected vary between the different studies (Table 3). This may in part be due to the different types of array platforms used, different sample pre-treatment procedures (e.g. microdissection versus whole tissue analysis), different analysis cut-off settings, but also the heterogeneous nature of the lesions tested concerning duration of existence and potential hrHPV type related differences.

Table 3: Overview of the aberrations obtained by CGH studies on CIN2/3.

<table>
<thead>
<tr>
<th>Study</th>
<th>Tissue</th>
<th>Aberration</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umayahara et al¹</td>
<td>33 CIN2/3</td>
<td>gains</td>
<td>3q26.1-q28 (48%); 1p (21%); 1q (21%);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11q23-qter (27%); 4pter-p15.1 (21%);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6p24-p22 (18%); 2q35-qter (18%)</td>
</tr>
<tr>
<td>Alazawi et al²</td>
<td>51 hgSIL</td>
<td>gains</td>
<td>1p (80%); 17q (47%); 20q (47%); 9q (45%); 1q (22%); 6p (22%); 3q (20%);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16q (18%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>losses</td>
<td>4q (53%); 6q (43%); 2q (33%); 17p (27%); 13q (25%); 5q (24%)</td>
</tr>
<tr>
<td>Kirchoff et al³</td>
<td>17 CIN3</td>
<td>gains</td>
<td>3q (35%); 18q (18%); 1p (18%); 1q (18%); 8q (18%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>losses</td>
<td>4q (24%); 13q (18%); 4p (18%); 6p (18%)</td>
</tr>
<tr>
<td>Heselmeyer et al⁴</td>
<td>19 CIN2/3</td>
<td>gains</td>
<td>X (21%)</td>
</tr>
<tr>
<td>Wilting et al⁵</td>
<td>46 CIN2/3</td>
<td>gains</td>
<td>20q12 (33%); 7q31.1-q31.2 (28%); 1p31.3-p21.1 (26%); 3q11.2-q29 (26%); 20p13-p11.21 (26%); 1q25.3-q32.1 (24%); 1q32.2-q44 (24%); 1p36.11-p35.2 (22%); 3p26.3-p26.1 (22%); 3p14.3-p14.2 (22%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>losses</td>
<td>17q23.3 (41%); 17p13.3 (35%); 4p16.3-p16.1 (33%); 19p13.11-q12 (33%); 11q13.3 (28%); 4q35.2 (26%); 4q31.21 (22%); 16q24.2-q24.3 (22%)</td>
</tr>
</tbody>
</table>

*note: chromosomal aberrations with reported frequencies of >15% are shown.
Thesis outline

Infection with hrHPV is the first step in the pathway towards cervical carcinoma. However, infection alone is not sufficient to drive malignant transformation and additional aberrations within the host cell genome are necessary, both at the epigenetic and the genetic level. Now that HPV-testing will become the primary cervical cancer screening tool, it is crucial to find suitable triage biomarkers to aid the risk stratification of hrHPV-positive women. For this, it is necessary to obtain better insight into the aberrations underlying disease development. Up to now, (epi)genetic aberrations have been examined in high-grade CIN, yet the reported results sometimes vary substantially, likely, at least in part, due to the heterogeneous nature of this type of lesion. Therefore, in this thesis the heterogeneous nature of high-grade CIN has been investigated at both the chromosomal and epigenetic level. The following questions were raised:

Chapter 2: How do chromosomal aberrations in a high-grade CIN relate to its duration of existence?

Using arrayCGH, it was investigated whether chromosomal aberrations are related to the duration of existence of a high-grade CIN, using duration of preceding hrHPV infection as a surrogate marker. This duration could be estimated using data of women participating in the POBASCAM cohort study. High-grade CIN with a relatively short-term preceding hrHPV infection (<5 years) were compared to those with a long-term preceding hrHPV infection (≥5 years). This indicated the presence of significantly more chromosomal aberrations in lesions of women with a long-term preceding hrHPV infection. This suggests that chromosomal profiling is capable of distinguishing high-grade CIN with a short-term progression risk in need of immediate treatment from those that could be managed by close surveillance.

Chapter 3: How do chromosomal aberrations in a high-grade CIN relate to the hrHPV type present?

Heterogeneity of high-grade CIN was also investigated in context of the hrHPV type present within the lesion. Chromosomal aberrations of high-grade CIN of women with a long-term preceding hrHPV infection (≥5 years) were examined. Lesions with HPV16 in general had fewer chromosomal aberrations compared to lesions with other high-risk types. Analysis of high-grade CIN with HPV16 versus HPV31 indicated a three-fold increase in the number of losses in lesions with HPV31. Losses at chromosomes 2q, 4p, 4q, 6p, 6q, 8q and 17p and gain at 1p and 1q were significantly more frequent in HPV31-positive lesions.

Chapter 4: Is it possible to identify driver genes in HPV-mediated carcinogenesis by analysis of focal chromosomal aberrations?

ArrayCGH analysis of high-grade CIN identified a number of focal chromosomal aberrations (<3 Mb in size). The small size of such aberrations facilitates the identification and validation of potential driver genes in the carcinogenic
process. The use of premalignant lesions instead of carcinomas further facilitates identification, due to the lower degree of genetic chaos and consequently fewer passenger aberrations. Expression of genes residing within the most frequently occurring focal aberrations were examined in two external mRNA expression microarray datasets. This revealed concurrent altered expression in high-grade CIN and/or cervical carcinomas compared to normal cervical samples for ATP13A3, HES1, OPA1, HRASLS, EYA2, ZMYND8, APOBEC2 and NCR2. In addition, functional roles of two candidate driver genes were determined via proliferation and migration assays. In this manner, EYA2 (located within a focal gain at 20q) and hsa-miR-375 (located within a focal loss at 2q35) were identified as actively contributing to HPV-induced carcinogenesis as an oncogene and tumour suppressor gene, respectively.

Chapter 5: Does promoter methylation of MAL result in gene silencing?

MAL was identified previously as the most significantly downregulated gene in cervical carcinoma. The mechanism underlying MAL silencing, its functional role in cervical carcinogenesis and the relevance of detecting MAL aberrations for risk-assessment of hrHPV-positive women was investigated. Expression of MAL was shown to be silenced by DNA methylation in HPV-immortalised cell lines and up to 93% in cervical carcinomas. In addition, its functional role was determined via proliferation, migration assays and anchorage-independent growth assays and indicated that MAL acts as a tumour suppressor. MAL promoter methylation increased with the severity of cervical lesion grade and could also be detected in cervical scrapes and may therefore be indicative of underlying high-grade CIN.

Chapter 6: How are methylation levels of biomarkers influenced by severity of underlying CIN disease and the duration of existence of high-grade CIN?

Combined detection of CADM1 and MAL promoter methylation in cervical scrapes was previously shown to be a promising triage strategy for risk-stratification of hrHPV-positive women with clinically relevant disease. Here, CADM1 and MAL methylation levels in cervical scrapes were examined in a screening cohort of hrHPV-positive women in relation to severity of cervical disease, as assessed by histology (≤CIN1 versus CIN2/3), either or not combined with cytology (normal versus abnormal). As a difference in the number of chromosomal aberrations was witnessed in high-grade CIN with respect to duration of preceding hrHPV infection (PHI), a surrogate of lesion age, this factor may also influence the levels of promoter methylation. Therefore, CADM1 and MAL methylation levels were examined in hrHPV-positive cervical scrapes of women with CIN2/3 following a short-term (<5 year) and long-term (≥5 year) PHI, respectively, as well as in women with carcinoma. CADM1 and MAL promoter methylation levels were found to be related to the degree and duration of underlying CIN disease and were markedly increased in case of cervical cancer.

Chapter 7: Summary, Discussion and Future perspectives.

This chapter contains an overview of the studies described in this thesis and discusses the reported findings and the importance of the gained insights.
Chapter 1

Reference List


General Introduction


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