Chapter 1

General introduction
Cervical Cancer

Cervical cancer is the third most common malignancy in women worldwide, with a total of 529,000 new cases and 274,000 deaths in 2008. Of these deaths, 88% occurred in developing countries, which most likely reflects a lack of well-organised screening programs \(^1\). Squamous cell carcinomas (SCCs; 80%) constitute most cases of cervical cancer, followed by adenocarcinomas (AdCA; 5-20%). Persistent infection with high-risk human papillomavirus (hrHPV) is causally involved in the development of both SCCs and AdCAs of the cervix \(^2,3\). It is estimated that about 80% of the sexually active women will experience at least one genital HPV infection at some point in their life, but fortunately most infections will be cleared by the immune system \(^4\). Even persistent infections do not automatically develop into cervical cancer, indicating that additional changes in the host cell are indispensable for this process.

Cervical cancer arises at or near the transformation zone (TZ), an area which is thought to be most susceptible to oncogenic influences of hrHPV types. The border between the columnar epithelium, located in the inner part of the cervix at the uterine site (endocervix), and the squamous epithelium, located at the outer part at the vaginal site of the cervix (ectocervix) is called the squamo-columnar junction (SCJ) (Figure 1). From puberty onwards the SCJ shifts from the outer part of the cervix into the direction of the more inner part of the cervix, thereby replacing the columnar epithelium from the endocervix by squamous metaplastic epithelium from the ectocervix. The TZ is the area with metaplastic squamous epithelium, which is clearly visible upon colposcopic inspection.

Figure 1. As females age, the location of the squamocolumnar junction (SCJ) on the cervix shifts. The replacement of the columnar epithelium from the endocervix by squamous epithelium from the ectocervix defines the transformation zone (TZ). Partially adapted from Herzog \(^5\).

Cervical Carcinogenesis

SCCs develop from hrHPV-positive non-invasive precursor stages, so-called cervical intraepithelial neoplasias (CINs), that are graded from 1 to 3 (Figure 2A)\(^6\). CIN1 corresponds to mild dysplasia, CIN2 to moderate dysplasia and CIN3 to both severe dysplasia and carcinoma in situ (CIS). CIN1 lesions are referred to as low-grade CIN and CIN2 and CIN3 as high-grade CIN. CIN lesions can regress spontaneously, but with increasing CIN grade the likelihood of regression decreases \(^7\). In about 80% of the hrHPV infected women, the virus is cleared without any apparent lesion development. In approximately 20% of the cases a persisting infection results in CIN lesions. Since only 5% of the CIN lesions, when left untreated, will eventually develop into cervical cancer, cancer is considered a rare complication of a hrHPV infection \(^8\). Precursor lesions of AdCA are less well defined, but adenocarcinoma in situ (ACIS) is a well-known immediate precursor lesion (Figure 2B)\(^9\).

Recently, Herfs et al. (2012) identified a discrete population of ~40 cuboidal epithelial cells, so-called squamo-columnar junction (SJC) cells, localized between the TZ and endocervical columnar cells. These cells are hypothesized to represent the progenitor cells of most, if not all, HPV-associated cervical carcinomas. These embryonal cells display a unique morphology and immunohistochemical staining pattern \(^10,11\). According to this hypothesis, most high-grade CIN and invasive carcinoma would arise from a hrHPV infection of these SCJ cells whereas infection of other parts of the TZ would give rise to non-progressive low-grade CIN at maximum (Figure 2C).

Cervical Cancer Prevention

Cervical cancer can be prevented by primary prevention, involving prophylactic vaccination, which was introduced in the Netherlands in 2009 \(^12\), and secondary prevention, which involves population-based cervical screening \(^12\).

Vaccines

Currently, two prophylactic vaccines for primary cervical cancer prevention are available. Cervarix® (GlaxoSmithKline) is a bivalent vaccine that protects against the two most common hrHPV types in cervical cancer, i.e. HPV16 and 18. Gardasil® (Merck), a quadrivalent vaccine, is directed against HPV16 and 18 as well as the low-risk HPV types 6 and 11, which are implicated in 75–90% of genital warts \(^13,14\). In the Netherlands vaccination by Cervarix® was introduced in 2009 and offered to 12-year-old girls with an initial catch-up vaccination for 13- to 16-year-old girls \(^15\). As the vaccines do not protect
against existing hrHPV infections and at present the coverage in the Netherlands is low, cervical screening will remain the most important prevention strategy for the majority of women for at least the next 20 years.

**Population-based Screening**

Cytomorphological examination of exfoliated cervical epithelial cells from the transformation zone, the so-called Pap-smears, was introduced in the Netherlands in the late 1970s. This test was developed by Papanicolaou and Traut in 1941 and is still used in population-based screening programs. In the Netherlands, women between the age of 30 to 60 are invited for a Pap smear taken by the general practitioner every five years and these smears are classified according to the CISOE-A system, see Table 1. Pap1 indicates normal cytology, Pap2 borderline dyskaryosis, Pap3a1 mild dyskaryosis, Pap3a2 moderate dyskaryosis, Pap3b severe dyskaryosis, Pap4 suspicion of CIS and Pap5 suspicion of invasive carcinoma. When borderline or mild dyskaryosis (Pap2/Pap3a1, hereafter referred as BMD) is diagnosed, women are advised to repeat the Pap test after 6 to 18 months. If BMD persists or progresses, women are referred to a gynaecologist for colposcopy. Given their high risk for high-grade CIN or worse, women with moderate dyskaryosis or worse (Pap3a2c) are directly referred to the gynaecologist for colposcopy.

**Table 1. CISOE-A classification.**

<table>
<thead>
<tr>
<th>S</th>
<th>O</th>
<th>E</th>
<th>Pap Description</th>
<th>Bethesda 2001</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Inadequate</td>
<td>Unsatisfactory for evaluation</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1–2</td>
<td>Normal</td>
<td>Negative for intraepithelial lesion or malignancy</td>
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<tr>
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<td>2</td>
<td>1–2</td>
<td>Normal</td>
<td>Atrophy, negative for intraepithelial lesion or malignancy</td>
</tr>
<tr>
<td>2–3</td>
<td>3</td>
<td>3</td>
<td>Borderline dyskaryosis</td>
<td>ASC-US/ASC-H</td>
</tr>
<tr>
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<td>4</td>
<td>3a1</td>
<td>Mild dyskaryosis</td>
<td>ASC-H/LSIL</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>3a2</td>
<td>Moderate dyskaryosis</td>
<td>HSIL</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>3b</td>
<td>Severe dyskaryosis</td>
<td>HSIL</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>4</td>
<td>Carcinoma in situ</td>
<td>AIS</td>
</tr>
<tr>
<td>8–9</td>
<td>7–8</td>
<td>9</td>
<td>Carcinoma</td>
<td>Squamous cell carcinoma</td>
</tr>
</tbody>
</table>

*Adapted from Bulk. Abbreviations: CISOE-A, C composition, I inflammation, S squamous epithelium, O Other abnormalities and endometrium, E endo-cervical columnar epithelium and A Adequacy of the smear; LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells cannot exclude HSIL; AGC, atypical glandular cells; AIS, endocervical adenocarcinoma in situ

Although population-based screening programs have led to a decrease in cervical cancer in developed countries, cytological screening has its drawbacks. While cytology-based screening has resulted in a decrease of SCCs, the incidence of AdCAs has remained the same or even increased in younger women (Figure 3). This is presumably due to the higher location of AdCAs and ACIS in the endocervical canal, which are therefore probably less prone to be represented in a standard specimen of exfoliated cells. Moreover, the decline in incidence of cervical SCC has levelled-off, which likely reflects poor reproducibility and only moderate sensitivity (at best 70%) of the Pap smear test for pre-cancerous lesions.
HPV testing

HrHPV is detected in up to 99.7% of the SCCs and 94-100% of the AdCAs of the cervix. In large randomized screening trials, testing for hrHPV has shown to have superior protection against cervical (pre-)cancerous lesions and cervical cancer compared to cytology. Therefore, cervical screening by primary hrHPV testing is now recommended by the Dutch Health Council and will be introduced in The Netherlands in 2016. However, only a fraction of hrHPV positive women will have or develop high-grade CIN or worse. Therefore, novel biomarkers are needed that can distinguish hrHPV positive women with high-grade disease from those with clinically irrelevant infections. These markers can be used to triage hrHPV positive women for colposcopy. Currently, cytology testing has emerged as the best available triage tool to identify hrHPV positive women in need of further gynaecologic examination, provided that it is performed at a high quality level as in the Netherlands. However, for hrHPV positive women with normal cytology at baseline it is critical to repeat cytology testing within one year to maximize the benefits of primary hrHPV testing in routine cervical cancer screening. Other proposed triage tools are HPV16 or HPV 16/18 genotyping, viral load assessment, HPV E6/E7 mRNA detection and p16INK4A immunostaining. Also, epigenetic silencing of tumour suppressor genes by DNA methylation in cervical (pre-)cancers provides disease biomarkers with great potential. DNA methylation can be easily detected in clinician-collected cervical scrape samples and self-collected cervico-vaginal specimens and has a high sensitivity for cervical cancer and high-grade CIN in both sample types. Moreover, the objective read-out of DNA methylation testing and the compatibility with HPV testing and self-sampling indicates that methylation markers are attractive triage markers, which allows for full molecular cervical screening in the near future.

**HUMAN PAPILLOMAVIRUS**

Papillomaviruses consist of double-stranded, circular DNA of approximately 7,900 bp in size, which contains a non-coding long control region (LCR) and eight open reading frames (ORFs), representing the early genes (E1, E2, E4, E5, E6 and E7) and the late genes (L1 and L2) (Figure 4A). HPVs belong to the family of *Papillomaviridae* and more than 100 different HPV types have been characterized (Figure 4B). A new HPV type is defined when more than 10% of the viral DNA sequence of E6, E7 and L1 differs from any other known HPV type. HPVs are categorized as cutaneous (beta- and gamma genera) or mucosal (alpha genus) types, based on their site of infection. Cutaneous types infect the epithelial cells of the skin, while mucosal types infect the mucosal lining of the mouth, throat, respiratory tract, and/or anogenital epithelium.
HPVs can further be subdivided into low-risk or high-risk, dependent on their capacity to cause lesions that can progress to invasive cell carcinomas. Most HPVs are low-risk and either do no cause disease, are linked to benign warts or low-grade intraepithelial neoplasias that do not undergo malignant transformation when left untreated. Some cutaneous HPV types, such as HPV 5, 8, 9, 12, 14, 15, 17, 19-25, 38 are associated with non-melanoma skin cancers, particularly those in patients with the hereditary disease epidermodysplasia verruciformis (EV). According to epidemiological and biological criteria 12 mucosal HPVs types have been classified as high-risk for development of cervical cancer (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) and eight as probable or possible high-risk (HPV26, 53, 66, 67, 68, 70, 73 and 82) 40. HPV16 and 18 are responsible for 70% of cervical cancers and about 50% of high-grade CIN lesions 41. The distribution of HPV types differs between SCC and AdCA. Particularly, the contribution of HPV18 to AdCA is higher than to SCC 42.

**VIRAL LIFE CYCLE**

HPV infections that result in production of new viruses are believed to start by infection of basal cells of the squamous epithelium 43. Host cell entry of HPV is suggested to be initiated by binding of the virus particle to the cell surface receptors heparin sulphate proteoglycans (HSPGs) 44,45. This binding results in conformational changes of the capsid proteins, followed by nuclear import of the DNA, though these processes are not entirely understood. The viral life cycle is coupled to the differentiation program of the infected epithelium 46. In undifferentiated basal cells, the HPV genome persists as a low copy number plasmid with the early genes expressed at low levels. Upon differentiation, viral gene expression gradually increases and the viral genome becomes replicated to a high copy number. Subsequently virus assembly occurs in the more differentiated epithelial cell layers, and new virus particles are released via shedded terminally differentiated cells. Viral replication as detected in low-grade CIN, a condition representing so called productive infections. This altered expression is reflected by an increased expression of the E6 and E7 oncoproteins in dividing cells and absence of expression of ‘late’ genes, such as L1 and L2. Expression of E6 and E7 is regulated by the LCR to which cellular transcription factors like Activator Protein 1 (AP1) and specificity factor 1 (Sp1) can bind, as well as the viral protein E2 47. The LCR contains four E2BS characterized by the specific sequence 5'-ACCN_GGT-3' 57. E2 can act either as a transcriptional repressor or activator, depending on which E2BS is bound. It has been suggested that deregulated expression of E6 and E7 in dividing cells may result from loss of E2 repression. One established mode of E2 downregulation is by viral DNA integration into the host cell DNA 48. When integrated, the HPV genome is often disrupted within or just upstream of the E2 ORF, thereby either affecting the continuity of that gene or uncoupling it from its promoter sequence 19. However, not all cervical lesions associated with a transforming infection harbour integrated viral DNA 40,41. Moreover, previous studies have often shown presence of an intact E2 ORF in cervical cancers, despite absence of E6 and E7 repression 42,43. Another event that may cause E6/E7 deregulation is DNA methylation, a well-known and critical regulator of transcription 44. Each E2BS contains one or two CpG dinucleotides, which are potential targets for DNA methylation. In vitro studies have shown that methylation of CpGs in the E2BS inhibits binding of E2 45, leading to loss of E2 repression of the E6 and E7 oncoproteins 46. Recent studies have demonstrated that the HPV genome becomes subject to DNA methylation during cervical carcinogenesis, though present data on site specific methylation are inconclusive 47 (see HPV methylation).

**HPV induced transformation**

Interference of E6 and E7 with cellular proteins disrupt cell cycle control and apoptosis 54,55,59,68. This disruption promotes chromosomal instability, enhancing cellular transformation. To date, multiple interaction partners for E6 en E7 have been identified, several of which will be discussed below. The best known binding partner of E6 is p53, as part of a trimeric complex with the cellular protein E6-Associated Protein (E6AP) ubiquitin ligase (Figure 5). This interaction results in degradation of p53 via the ubiquitin-dependent proteolysis pathway 69-71. P53 degradation abolishes cell cycle control and apoptosis, allowing cell division in the presence of DNA
E6 is also able to inhibit p53-independent apoptotic pathways by interacting with other proteins involved in apoptosis, like BAK, BAX, c-Myc, pro-caspase-8 and Fas-associated death domain (FADD) all of which are inactivated through the ubiquitin proteosome pathway. PDZ (PSD95/Dlg/ZO-1) domain containing proteins, including MUPP-1, hSCRIB and Dlg, can also be bound by high-risk HPV E6. These interactions appear to be essential for the normal viral life cycle, though their exact role during cervical carcinogenesis needs to be further elucidated.

For cells to become immortal, transcriptional activation of the gene encoding human Telomerase Reverse Transcriptase (hTERT), the catalytic subunit of the telomerase enzyme complex, is critical. Primary cells undergo a finite series of population doublings followed by a senescence phase. Upon inactivation of p53 and pRb pathways by E6 and E7, cells acquire an extended lifespan and may become immortal. Immortalization has been linked to telomerase activation resulting from increased expression of the catalytic subunit hTERT. HPV16 E6 has been demonstrated to activate transcription of hTERT by different mechanisms, though exact mechanisms remain controversial. Previous reports have indicated that hTERT activation correlates with the ability of E6 to bind E6AP and c-myc, allowing c-myc to be more active at the hTERT promoter. Other studies indicate that E6 can upregulate hTERT expression by binding and degradation of NFX1-91, thereby inhibiting the function of the mSin3A/histone deacetylase (HDAC) complex at the hTERT promoter. A more recent study reports E6 binding to hTERT protein itself, which could lead to hTERT stabilization and/or change its localization in the cell.

E7 can interact with the pocket protein family members pRb, p130 and p107, thereby interfering with the control at the G1/S transition of the cell cycle. This results in de-repression of E2F transcription factors, which in turn leads to cell cycle progression towards the S-phase entry. E7 deregulation in proliferating cells can be identified by an upregulation of the cdk4 inhibitor p16INK4A, which is supposed to be a marker for a transforming infection. Normally, this inhibitor is regulated by an Rb-dependent negative feedback loop and continuous inactivation of pRb by E7 has been suggested to contribute to increased expression of p16INK4A in high-grade CIN. However, more recently, it has been found that p16INK4A upregulation can be accomplished in a Rb-independent manner through upregulation of the histone demethylase KDM6B by E7. Additionally, E7 can promote G1 to S phase transition by interacting with p21, thereby blocking the inhibition of cyclin E/cdk2 activity, leading to pRb phosphorylation. E7 can also deregulate cell cycle control through interactions with HDACs and the DNA methyltransferase DNMT1, thereby modulating chromatin remodelling and accessibility of cell cycle regulatory proteins, such as the E2F promoter.

Figure 5. Schematic overview of the mechanisms of E6 and E7 interference with apoptosis and cell cycle control. Formation of an E6-E6-Associated Protein (E6AP)-p53 trimeric complex results in p53 degradation, thereby inhibiting apoptosis. E6 also inhibits apoptotic signalling by interacting with pro-caspase-8, BAK and BAX and Fas-associated death domain (FADD) and activates transcription of human Telomerase Reverse Transcriptase (hTERT) necessary for immortalization. E7 can subvert G1-S arrest through inhibition of the retinoblastoma protein (pRb) and constitutive activation of E2F target genes. Activation of KDM6B by E7 and inhibition of pRb results in increased expression of p16INK4A. Cell cycle control is further deregulated through inhibition of cyclin-dependent kinase inhibitors (such as p21).

In order to gain more insight into HPV-mediated transformation of epithelial cells, the use of in vitro model systems, in which primary keratinocytes are immortalised by HPV16 or HPV18, have proven to be very useful. These studies have shown that HPV-mediated transformation can be subdivided into at least four different stages, as reviewed by Steenbergen et al. (Figure 6). With a maintained expression of E6 and E7, HPV transfected keratinocytes gain an extended life span, a.o. by inactivation of p53 and pRb. By induction of hTERT, cells can acquire an immortal phenotype. Continued culturing of these cells results in additional (epi)genetic alterations that may ultimately lead to anchorage independent growth conditions and tumourigenicity in immunocompromised mice.

Alterations that are likely to contribute to HPV-induced transformation include mutations, allelic loss (loss of heterozygosity), chromosomal alterations and/or epigenetic alterations. Currently, relatively few mutations in tumour suppressor genes and oncogenes have been described for cervical cancer. The highest mutation rate is observed for p16.
Examples of these modifications include acetylation, methylation, ubiquitination, phosphorylation and glycosylation. The amino acids lysine (K), serine (S), tyrosine (Y) and arginine (R) are targets for these modifications. Combinations of modifications are thought to constitute a code, the so-called histone-code, whereby combinations of histone modifications have specific meanings. Most functional data of histone modifications are involved in control of transcription. In Table 2 a couple examples of histone modifications and their effects on gene expression are listed.

Epigenetics describes heritable changes in gene expression or cellular phenotype, caused by mechanisms other than changes in the underlying DNA sequence. Epigenetic mechanisms include histone modifications, DNA methylation, action of noncoding RNAs and other complementary mechanisms controlling higher order chromatin organization within the cell nucleus. The basic functional unit of chromatin is the nucleosome, which contains 146 base pairs of DNA, which is wrapped around a histone octamer. Each octamer contains two each of histones H2A, H2B, H3, and H4. Linker histones, such as H1, can further package nucleosomes to form higher-order chromatin structures. Chromatin can be subdivided into heterochromatin, a tightly packed form of DNA, which primarily contains repressed genes, and euchromatin, a lightly packed form of DNA, which contains most of the actively expressed genes.

Histone modifications

The tails of histone proteins can undergo many different posttranslational modifications, which alter the DNA-histone interactions and, therefore, affect high-order chromatin.

Table 2. Histone modifications in transcription regulation.*

<table>
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<tr>
<th>Modification</th>
<th>Position</th>
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<td>Active</td>
</tr>
<tr>
<td></td>
<td>K9</td>
<td>Repressive</td>
</tr>
<tr>
<td></td>
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<td>Repressive</td>
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<td></td>
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<td></td>
<td>K79</td>
<td>Active</td>
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<tr>
<td>Acetylation</td>
<td>H4 K20</td>
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</tr>
<tr>
<td>Ubiquitination</td>
<td>H2A K119</td>
<td>Repressive</td>
</tr>
<tr>
<td></td>
<td>H2B K120</td>
<td>Active</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>H3 S10</td>
<td>Active</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>H3 S10</td>
<td>Repressive</td>
</tr>
</tbody>
</table>

* Partially adapted from Li.

Acetylation of histones neutralizes the positive charge of lysines, creating a more open chromatin architecture. This modification is catalyzed by histone acetyltransferases (HATs) and reversed by HDACs. Histone methylation does not affect the overall charge of the histone tail, suggesting that it does not alter chromatin structure. Rather, this covalent modification probably serves as a recognition motif for the binding of chromatin-associated proteins in order to maintain local regions of active or inactive chromatin. Histone arginines can be mono- or dimethylated, while lysines can become mono-, di- or trimethylated. Almost all histone methyltransferases (HMTs) contain a SET domain that harbours the enzymatic activity. Initially, histone methylation was considered a stable, static modification, though recently, histone demethylases, such as LSD1 and JHDM1 have been identified. Ubiquitination occurs on histones H2A and H2B. Ubiquitin itself is a 76-amino acid polypeptide. Histone ubiquitination results in a large covalent
modification, though it is still a highly dynamic one. The modification is removed via the action of isopeptidases called de-ubiquitin enzymes (DUBs) and this activity is important for both gene activity and silencing. The addition of negatively charged phosphate groups to histone tails neutralizes their basic charge and is thought to reduce their affinity for DNA. DNA methylation involves the covalent addition of a methyl group (-CH₃) to cytosines which precede guanines in the DNA sequence, called CpG dinucleotides (Figure 7A). CpGs are usually located in short stretches of CpG-rich sequences, known as CpG islands, which can be found in approximately 60% of human gene promoters. CpG islands are unmethylated in active genes, whereas silenced genes are characterized by methylation of the promoter region (Figure 7B).

Mechanisms of DNA methylation

DNA methylation involves the covalent addition of a methyl group (-CH₃) to cytosines which precede guanines in the DNA sequence, called CpG dinucleotides (Figure 7A). CpGs are usually located in short stretches of CpG-rich sequences, known as CpG islands, which can be found in approximately 60% of human gene promoters. CpG islands are unmethylated in active genes, whereas silenced genes are characterized by methylation of the promoter region (Figure 7B). In the remainder of the mammalian genome, the CpG content is low and the majority of these are methylated. DNA methyltransferases are responsible for DNA methylation, using S-adenosyl methionine (SAM) as a methyl donor. During development, the genome undergoes a series of epigenetic reprogramming events. Novel DNA methylation patterns are established by the de novo DNA methyltransferases DNMT3A and DNMT3B. The maintenance DNA methyltransferase, DNMT1, ensures that this DNA methylation distribution is stably inherited throughout development.

During carcinogenesis the DNA methylome changes. Globally hypomethylation occurs, which contributes to genomic instability and to activation of silenced oncogenes. On the other hand, local hypermethylation is also known as a critical hallmark of cancer cells. Hypermethylation usually occurs in CpG islands, resulting in transcriptional silencing of genes that could act as tumour suppressors. Interestingly, recent studies have found that regions with a relatively low CpG density flanking CpG islands (up to 2 kb distant), termed CpG island shores, show tissue- and cancer-specific methylation alterations. This suggests that CpG island shore methylation may also be involved in tissue differentiation, epigenetic reprogramming and cancer development.

HPV methylation

Human genomes are potential targets of invasion by viruses and transposable elements. To prevent the genome from being completely taken over by molecular invaders, the genome has to recognize invaders and raise a specific “immune response” against them. One proposed strategy is to suppress viral genes and transposons by selective methylation of the viral and transposon sequences. The HPV genome has been shown to become subjected to DNA methylation during disease progression. Most studies have focussed on DNA methylation of the HPV16 genome and to a lesser extent HPV18, while only a few studies have addressed other high risk HPVs. HPV16 has in total 113 CpGs, which are unevenly distributed throughout the viral genome. Association between methylation at specific CpGs and cervical disease has shown considerable variation in different studies.
Hypermethylation of CpGs within the L1 gene (and L2 gene) with increased lesion severity seems to be relatively consistent across studies. This indicates that L1 methylation may provide a candidate biomarker, though the functional consequence of methylation within this gene is unclear.

Methylation of the LCR is of functional interest, as it is able to regulate transcription of the E6 and E7 oncoproteins (see Transforming infection). Some studies have found increased methylation in the LCR, especially at the E2BS, while others reported decreased methylation associated with high-grade CIN. A possible explanation for these discrepant findings may relate to the presence of multiple HPV copies in a lesion and/or cell lines. The cervical cancer cell line CaSkI contains approximately 400-600 copies of HPV16 DNA which are densely methylated, while SiHa cells only contain 1-2 copies which show little to no methylation. Van Tine et al. (2004) indicated that only one or a few of the HPV16 copies in CaSkI cells are actively transcribed, whereas the remaining copies are silenced by methylation. Upon treatment with a DNA demethylating agent, 5-azacytidine, transcription from the silent HPV copies became active. Other explanations for the inconsistency between the HPV methylation studies may be due to limited sample sizes, differences in detection methods, sample series and/or CpGs analyzed. It has been postulated that these changes in HPV methylation may serve as predictive or diagnostic biomarkers for HPV-positive women at risk for cervical cancer. To determine whether HPV methylation can in the future be used as a diagnostic marker, more in-depth analysis is required with larger sample sizes and including more CpGs.

Methylation of host cell genes

During the last years, many studies have identified altered methylation of promoter regions of tumour suppressor genes, as reviewed by Wentzensen et al. and Woodman et al. In total, over 68 genes have been identified to be subjected to methylation alterations during cervical carcinogenesis. Wentzensen et al. reported comparable methylation frequencies across different studies (>5) for only three genes, CADM1, DAPK1 and RARB. The discrepancies for other genes might in part be explained by methodological differences (see DNA methylation detection). CpGs tested and assay thresholds. The DNA methylation targets could serve as molecular markers of cervical (pre)malignant disease. Most studies in cervical cancer focused predominantly on SCC, and most epigenetic biomarkers currently under study have been deduced from these data. It is unclear to what extent the results obtained on cervical SCC can be translated to AdCA. Previous studies based on array-comparative genomic hybridization (arrayCGH) and Methylation Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) indicate that AdCA and SCC display different (epi)genetic profiles. Analysis of DNA promoter methylation patterns that differ between SCC and AdCA may result in AdCA specific epigenetic biomarkers.

The most promising methylation markers have a clearly defined role in the development of cervical cancer. For instance, CADM1 and MAL are frequently methylated in high-grade CIN or worse and have shown to influence proliferation, anchorage dependent growth, tumourigenicity and/or migration capacity of HPV transformed cells. Biomarkers based on detection of DNA methylation alterations are particularly interesting, as previous studies have indicated that these alterations can easily be detected in cervical scrapings in women with underlying cervical disease. Using cervical scrapings of referral populations, sensitivities of over 80% for high-grade CIN were obtained with marker panels SOX1-PAX1, SOX1-LMX1A, SOX1-NKX6-1, PAX1-LMX1A, PAX1-NKX6-1, LMX1A-NKX6-1, LMX1A-E2F1 and CADM1-MAL. Similar results were obtained for the CADM1-MAL marker panel in a population-based screening cohort.

DNA methylation detection methods

Over the last years, multiple methods have been developed to determine the methylation status of CpGs. These techniques are each based on one of the following three principles: (1) cleavage of DNA with methylation-sensitive restriction enzymes (MRE); (2) sodium bisulfite conversion of DNA; and (3) affinity purification to enrich for methylated regions. For each of these principles a couple of examples will be given below. The method of choice is mainly dependent of the aim of the methylation study, but also on equipment and expertise of the laboratory and the quality and quantity of the samples. The best known MRE is HpaII, which recognizes the sequence 5’ – CCGG – 3’ and only cuts when the sequence is unmethylated, while its isoschizomer MspI is insensitive for DNA methylation. Limitations of MRE digestion are risk of incomplete digestion resulting in false positives and analysis being restricted to CpGs within the recognition site of the MRE. The latter can be overcome when frequent cutters are used in parallel. MRE-digestion can be used to determine genome-wide DNA methylation patterns when combining methylation-dependent cleavage with sequencing-based methods. One example is Methylation-Specific Digital Karyotyping (MSDK), where DNA is first cleaved with the MRE Ascl, followed by cleavage with the frequent cutter Ncol. With the use of adapters, DNA molecules are PCR amplified, followed by Sanger sequencing. Other sequencing-based methods make use next-generation sequencing on the Illumina platform, like HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP)-seq and Methyl-seq. In these assays genomic DNA is cleaved with HpaII and MspI separately. MspI is used as a reference control allowing for some degree of methylation quantification.
Treatment of DNA with sodium bisulfite results in conversion of unmethylated cytosines into uracils, while leaving methylated cytosines unaffected. This allows for DNA methylation mapping at single base resolution, which can be detected by PCR amplification and sequencing. Upon PCR amplification of bisulfite treated DNA, the uracil is replaced by a thymine. There are two main alternatives to determine DNA methylation alterations by PCR, namely Methylation-Specific PCR (MSP), using primers containing CpGs, and Methylation-Independent PCR (MIP), using primers that do not overlap CpGs. With MSP, the primers can be completely complementary to methylated sequence or unmethylated sequence. PCR products can be detected by gel electrophoresis. With the addition of a hydrolysis probe, the conventional MSP can converted to a quantitative format, qMSP. This method has for example been used to measure DNA methylation levels of CADM1, MAL and hsa-miR-124 in cervical cancers. Methylation-specific high resolution melting (MS-HRM) makes use of MSP primers in combination with dyes that are fluorescent when incorporated into double-stranded DNA. Both qMSP and HS-HRM have similar analytical sensitivities and can detect as little as 0.1–1.0% of methylated DNA in a background of unmethylated DNA. With the use of MIP primers DNA is amplified regardless of its methylation status. DNA methylation can be determined by subcloning of the PCR-product followed by Sanger sequencing. A limitation of Sanger bisulfite sequencing is its low sensitivity. As usually a limited number of clones (<20) are analysed the sensitivity of this method is around 10%.

This method does not require cloning of the bisulfite-treated, amplified DNA into bacterial expression vectors and it provides quantitative data. Still, the sensitivity for bisulfite pyrosequencing is approximately 5%. Increased sensitivity rates, comparable to that seen for qMSP analysis, can be accomplished by ultra-deep bisulfite sequencing. This method is based on massive parallel sequencing-by-synthesis and can directly sequence over a hundred PCR products of bisulfite treated DNA in a single sequencing run. Though bisulfite conversion is used in the majority of current techniques for analysis of DNA methylation, it fails to discriminate between DNA methylation and DNA hydroxymethylation, the so-called ‘sixth base’. 5-Hydroxymethylcytosine (5hmC) is formed through the action of TET family of dioxygenases (TET1, TET2 and TET3), which utilize oxygen to transfer a hydroxylgroup to 5-methylcytosine (5mC). Bisulfite reacts with the hydroxymethyl group by converting it into 5-methylenesulfonate. Treatment with sodium bisulfite does not lead to deamination, as seen by unmethylated cytosines, making discrimination between 5mC and 5hmC impossible. The biological consequence between 5mC and 5hmC modifications differ. For example, certain DNA-methyl binding proteins do not to bind 5hmC, which may affect the transcriptional activity of genes.

Moreover, ShmC prevents DNMT1-mediated methylation, which will interfere with methylation maintenance during cell division and may lead to passive demethylation. Also active demethylation has been linked to ShmC, which is suggested to be mediated by base excision repair. Several methods have been developed to distinguish between 5mC and 5hmC. One approach makes use of the ability to oxidize ShmC to 5-formylcytosine (5fC) with potassium perruthenate (KrO4), enabling bisulfite conversion of 5fC to uracil. Another approach is based on glycosylation of 5hmC. This is achieved by ShmC-glycosyltransferase using uridine diphosphoglucose as co-factor, followed by enzymatic cleavage with glycosylation sensitive restriction enzymes and PCR amplification. Immunoprecipitation of methylated DNA with antibodies that are specific for methylated cytosines could also be used to discriminate between 5mC and ShmC. Examples are Methylated DNA immunoprecipitation (MeDIP) or the use of methyl-binding domain (MBD) proteins. MeDIP and MBD have both been coupled with DNA micorarrays, MeDIP-chip and MBD-chip, to obtain relative methylation levels at loci represented on the array. Coupling these methods with next-generation sequencing, MeDIP-seq and MBD-seq, allows for genome-wide assessment of DNA methylation. Li et al. compared these two methods with whole genome bisulfite-sequencing (BS-seq) and found that MedP-seq and MBD-seq are complementary strategies, with MeDIP-seq being more sensitive to highly methylated, high-CpG densities and MBD-seq more sensitive to highly methylated, moderate-CpG densities. Limitations of MeDIP and MBD are the need for substantial amounts of fresh genomic DNA and the absence of information on individual CpGs, as only DNA fragments containing multiple methylated CpGs are captured by the antibodies/proteins.

**Outline of thesis**

Infection with hrHPV is the primary risk factor for the development of cervical cancer. However, additional (epi)genetic alterations in the host cell genome are indispensable for malignant transformation. More insight into these (epi)genetic alterations can further improve our knowledge about HPV-mediated carcinogenesis and may enable further stratification of hrHPV-positive women at risk for developing cervical cancer. As outlined before, previous studies in our research group have indicated a number of highly frequent DNA methylation alterations in cervical carcinomas and precursor lesions, i.e. MAL, CADM1 and hsa-miR124-2. In this thesis we aimed to optimize DNA methylation detection methods and gain further insight in the epigenetic events, not only in the host cell genome, but also in the HPV16 genome itself, that occur during cervical carcinogenesis. Moreover, we determined the biological relevance of one of our
newly identified genes that was hypermethylated in HPV-transformed cells. Towards accomplishment of this aim, the following questions were raised.

Chapter 2: Can analysis of methylation marker combinations be simplified to allow high-throughput analysis of clinical samples? QMSP for CADM1, MAL, hsa-miR124-2 and the reference gene ACTB were combined in a single reaction. Upon optimizing primer sequences and concentration and comparison of various multiplex TaqMan buffer systems, we developed a multiplex qMSP. The multiplex qMSP showed the same analytical performance as the singleplex qMSPs. A strong correlation between the obtained normalized ratios of the singleplex and multiplex qMSPs on cervical scrapes was found for all three markers.

Although these three methylation markers are attractive triage markers for early detection of cervical cancer, they do not discriminate between SCC and AdCA. In search for AdCA specific biomarkers we focussed on the WNT/β-catenin signaling pathway, which is frequently deregulated in human AdCAs.

Chapter 3: Which DNA methylation markers can specifically detect cervical AdCAs or its precursor lesion ACIS? MSP targeting negative modulators of the WNT/β-catenin signalling pathway revealed frequent methylation of DKK3, SFRP2, SOX17 and WiFi1 in cervical AdCAs. The methylation frequencies and levels increased with severity of cervical disease. Methylation frequencies of DKK3 and SFRP2 were significantly higher in AdCA compared to SCC, while SOX17 methylation frequency was significantly higher in SCC than AdCA. Methylation of WiFi1 was common in both AdCA and SCC. When tested on HPV-positive cervical scrapings, DKK3 and SFRP2, detected all women with underlying ACIS/AdCA.

Since previously identified methylation markers do not detect all high-grade CIN or worse, there is still need for better and/or additional biomarkers. Mechanisms that contribute to the deregulated expression of the viral oncoproteins E6 and E7 could provide biomarkers with great diagnostic value. One such mechanism could be methylation of the E2BSs in the viral LCR which regulate transcription initiation of E6 and E7. We therefore examined the frequency of HPV 16 E2BS methylation in high-grade CIN and SCC.

Chapter 4: At what frequency does methylation of the HPV16 E2BSs occur in high-grade CIN and SCC? A novel DNA methylation detection method was developed to determine the frequency of E2BS methylation of HPV16, based on MIP in combination with the Luminex® xMAP™ system. All SCC showed methylation of at least one E2BS, compared to 58% of HIGH-GRADE CIN lesions and 24% of controls. E2BS3 may have diagnostic potential since it was found the be the most frequently methylated E2BS in high-grade CIN (47%) and occurred proportional to severity of cervical disease.

Given that E2BS methylation is a rather late event in cervical carcinogenesis, a continuation of our search for additional biomarkers was needed using a genome-wide approach.

Chapter 5: Which DNA methylation events contribute to a transforming hrHPV infection? With Methylation-specific digital karyotyping (MSDK) 184 locations displayed methylation alterations associated with a transforming hrHPV-infection. Thirty-four of these genes showed reduced expression in cervical carcinomas. For 12 genes (CLIC3, CREB3L1, FAM19A4, LFNG, LHX1, MRC2, NNX2-8, NPTX-1, PHACTR3, PRDM14, SOST and TNFSF13) specific methylation in HPV-containing cell lines was confirmed using MSP analysis. Subsequent analysis of FAM19A4, LHX1, NNX2-8, NPTX-1, PHACTR3 and PRDM14 in cervical tissue specimens showed increasing methylation levels for all six genes with disease progression. All genes were found to be frequently methylated in cervical carcinomas, with highest frequencies (up to 100%) seen for FAM19A4, PHACTR3 and PRDM14.

Most promising methylation markers are those that have a clearly defined role in the development of cervical cancer. To date, little knowledge exists on the role of some hypermethylated genes, such as PRDM14, in cancer and the biological relevance of PRDM14 methylation in HPV-induced carcinogenesis remains to be established.

Chapter 6: What is the biological relevance of PRDM14 methylation-mediated silencing in cervical carcinogenesis? By treatment of the cervical cancer cell line SiHa with the demethylating agent 5-aza-2’-deoxycytidine (DAC), we showed that PRDM14 methylation was inversely correlated with its expression. Ectopic PRDM14 expression in SiHa cells resulted in apoptosis, as measured by Annexin-V/PI FACS analysis, which was (directly) linked to upregulated NOXA and PUMA expression.

In Chapter 7 a summary and discussion are presented based on all data presented in this thesis.
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Chapter 1

General introduction


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