Altered DNA methylation during HPV-induced cervical carcinogenesis

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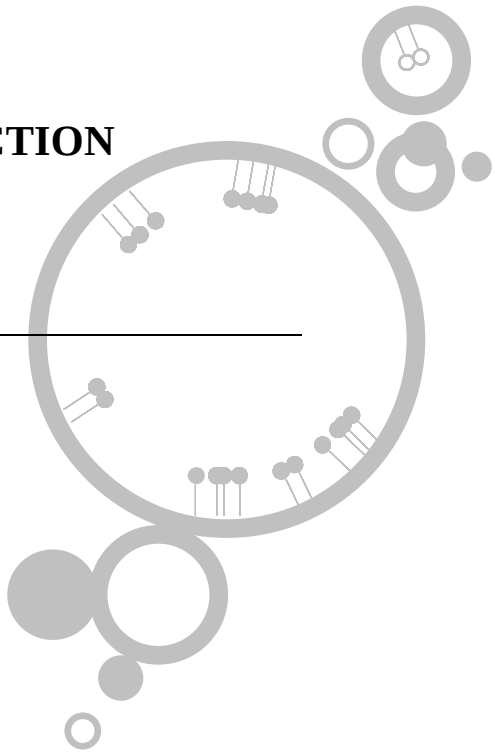
CHAPTER 1

GENERAL INTRODUCTION



CERVICAL CANCER

Epidemiology of cervical cancer

Cervical cancer is the second most frequent cancer amongst women worldwide, which is for the most part due to the high incidence of this disease in developing countries without well-organised cervical screening programs. Several types of cervical cancer can be distinguished based on histology. Squamous cell carcinoma (SCC) is the most common type of cervical cancer (about 80%), followed by adenosquamous carcinoma and adenocarcinoma (AdCA) (together 10-20%) and small cell carcinoma (<5%).

Population-based cervical cancer screening in developed countries has lead to a reduction of the frequency of cervical cancer of about 60% since 1960 [1-5]. Yet, epidemiological studies designate that even though the incidence of SCCs has drastically decreased, the frequency of AdCAs has remained the same or has even increased in developed countries [6-8].

While the age-standardised rate of invasive cervical cancer is at maximum 11.3 per 100,000 women in more developed countries, it is on average 18.7 per 100,000 women in developing countries with a peak incidence of 44.3 per 100,000 women in Middle Africa [9]. In The Netherlands the age-standardised rate of invasive cervical cancer in 2007 was 7.9 per 100,000 women with an age-standardised mortality rate of 1.9 [10].

Anatomy of the uterine cervix

The uterine cervix, the lower portion of the uterus, is positioned at the transition from the uterus to the vagina. The outer part of the cervix at the vaginal side (ectocervix) is lined with stratified non-keratinising squamous epithelium, whereas the inner part of the cervix at the uterine side (endocervix) is covered with a single layer of columnar, mucus-secreting epithelial cells (Figure 1). The

boundary between squamous epithelial cells and columnar epithelial cells is identified as the squamo-columnar junction (SCJ). In young women, the SJC is located on the ectocervix. During puberty, metaplastic changes at the SCJ result in the replacement of columnar epithelium by squamous epithelium. As a result of this physiological process, the SCJ shifts from the ectocervix towards the endocervix. The metaplastic area between the original and the new SCJ is named the transformation zone, which is assumed to be most susceptible to oncogenic influences of certain human papillomaviruses (HPVs) [11] and cervical cancer is believed to arise from this particular zone.

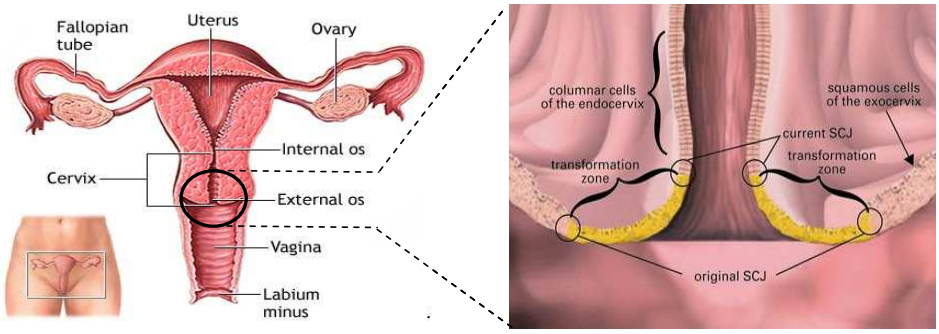


FIGURE 1. The uterus, cervix and transformation zone. Partially adapted from [12].

Cervical cancer and its precursor lesions

Cervical SCCs develop through pre-malignant precursor lesions, referred to as cervical intraepithelial neoplasia (CIN). These histologically distinct precursor lesions are classified from grade 1 to grade 3, based on progressive atypia of epithelial cells. CIN1 lesions, in which up to the lower 1/3 of the entire thickness of the epithelium shows dysplastic modifications, correspond to mild dysplasia. CIN2 lesions, in which up to 2/3 of the epithelium is engaged, are equivalent to moderate dysplasia. CIN3 lesions (in which involvement includes 2/3 to the entire thickness) comprise severe dysplasias and carcinomas *in situ* (CIS) (Figure 2) [13]. To reflect their relative risk of progression to cervical cancer, CIN1 lesions are referred to as low-grade CIN, and CIN2 and 3 lesions together as

high-grade CIN. As a result, existing practice is to treat women with high-grade CIN lesions, preferably by a Large Loop Excision of the Transformation Zone (LLETZ), to avert cervical cancer [14].

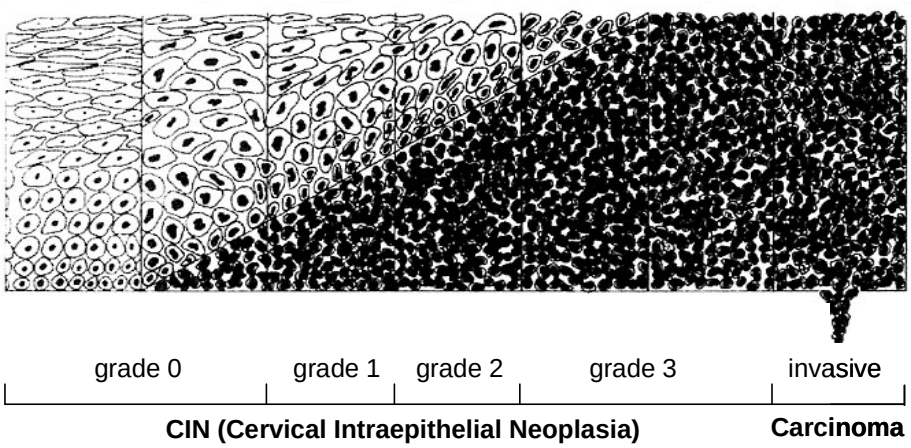


FIGURE 2. Schematic representation of cervical squamous precursor lesions relative to normal epithelium and invasive carcinoma. Adapted from [15].

Population-based cervical screening programs: cytomorphology

Existing population-based screening programs traditionally utilise the so-called Pap test, originally described by Papanicolaou and Traut (1941), in which smears of exfoliated cervical epithelial cells derived from the transformation zone are cytomorphologically examined [16]. Diverse cytomorphological classification systems are applied in various countries. In The Netherlands, a population-based screening program based on using the Pap test on a cervical scraping, was introduced in the late 1970s. Presently, women between 30 and 60 years of age are invited every 5 years to undergo a routine Pap scraping, performed by a general practitioner. For cytomorphological evaluation of the Pap scrapings, the CISOE-A scoring system is used [17,18], eventually resulting in a classification from Pap1 to Pap5 (inappropriate scrapings are classified as Pap0). Pap1 designates normal cytomorphology, Pap2 indicates borderline dyskaryosis, Pap3a1 points to mild dyskaryosis, Pap3a2 specifies moderate

dyskaryosis, Pap3b designates severe dyskaryosis, Pap4 indicates suspected of CIS, and Pap5 is suspected of at least micro-invasive cancer. Women with a Pap2/3a1 scraping (also referred to as borderline or mild dyskaryosis (BMD)) are not directly referred to a gynaecologist, since only about 10% of them have underlying high-grade CIN lesions and consequently, direct referral would result in a substantial overcall. Instead, these women are advised to have a repeat scraping taken after 6 and 18 months and they are only referred in case of BMD persistence or cytomorphological progression. Women with a Pap3a2 scraping (>BMD) or worse are immediately referred to the gynaecologist for colposcopy [17,18]. Although introduction of cytomorphology-based screening programs have seriously reduced the incidence of cervical cancer [3-5], screening by cytomorphology has several limitations. Cytomorphological examination of Pap scrapings is subjectively biased and has a rather low sensitivity, leading to a substantial false-negativity rate. Conversely, only a minority of women with BMD cytomorphology outcomes will have or develop high-grade CIN lesions resulting in a surplus of follow-up visits in this category of women [19,20].

HUMAN PAPILLOMAVIRUS

Human papillomavirus and cancer

Powerful molecular and epidemiological studies support the necessary and causal involvement of a persistent infection with a high-risk type of the sexually transmittable human papillomavirus (hrHPV) in the pathogenesis of cervical cancer [21]. Accordingly, hrHPV can be identified in approximately 99.7% of cervical SCCs and 94-100% of AdCAs of the cervix [22-25]. However, while cervical hrHPV infections are found relatively frequent in sexually active, particularly younger women, cervical cancers are relatively rare and for that reason should be considered an uncommon complication of an hrHPV infection

[26]. Evidence exists that both the extent of tolerance by the immune system and supplementary (epi)genetic events are required for malignant progression.
Although less outstanding, hrHPV has also been implicated in a subset of SCCs positioned in other parts of the anogenital tract, for instance anal, vulvar and penile carcinomas, as well as in SCCs originating in the head and neck region (HNSCCs), predominantly of oral and oropharyngeal origin. The number of cases associated with HPV is estimated to be in the range of 70-100% in anal and basaloid warty vulvar carcinomas [27-29], 60% in vaginal carcinomas [30], 30-40% in penile carcinomas [31-33] and 15-35% in oral/oropharyngeal HNSCCs [34-37].

General characteristics of HPV

Papillomaviruses are small double-stranded DNA viruses belonging to the family of Papillomaviridae [38]. Papillomaviruses are strictly epitheliotrophic and can be subdivided in cutaneous and mucosal types, on basis of their privileged site of infection. While the cutaneous HPV types predominantly infect skin, infections of mucosal types can be found in the epithelial linings of the anogenital, respiratory and upper digestive tract [21,39,40].
The HPV genome, which is approximately 7.9 kilo base pairs in size, can be separated into an early region, encoding proteins essential for viral replication (E1, E2, E4, E5, E6 and E7), and a late region, encoding the major and minor viral capsid proteins (L1 and L2, respectively). If the genomic sequence of the *E6*, *E7* and *L1* region show less than 90% sequence homology to any other known HPV type, a new HPV type is characterised. A homology between 90 and 98% determines a new HPV subtype and a homology between 98% and 100% defines an intratype variant [38]. Up to now, more than 130 HPV types have been acknowledged.
The majority of HPV types, the so-called low-risk types, are linked to benign wart-like lesions that often revert without treatment and reveal no risk of malignant transformation. Alternatively, a subset of mucosal HPVs may ultimately cause malignant transformation of the infected epithelium and these types are therefore designated as high-risk (hr) types. Pooled analysis of world-

wide case-control studies classified 15 HPV types as high-risk (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), while another three were considered potentially high-risk (HPV26, 53, and 66) [41]. HPV16 is predominant in HPV-associated carcinomas originating from mucosal linings of all anatomical sites. Although epidemiological studies indicate that the oncogenic potential varies between hrHPV types, functional confirmation using *in vitro* transformation studies still remains to be established, particularly for the uncommon hrHPV types.

The viral life cycle

The viral life cycle of HPV is closely related to the differentiation process of the infected epithelium, and the HPV virus largely depends on the host cell replication machinery for its viral DNA synthesis.

A productive HPV infection is initiated after viral particles have gained access to the epithelial basal layer and, following attachment to proteoglycans of the basement membrane and subsequently to secondary receptors on the cell surface, have entered the basal cells [42,43]. In these cells, only low levels of viral early gene activity is present, sufficient to facilitate genome preservation by co-replication with the host cell genome. During cellular differentiation, the viral differentiation-dependent promoter is up-regulated, resulting in increased levels of viral proteins necessary for viral genome amplification [44]. The proteins encoded by the viral genes *E1*, *E2*, *E4* and *E5* are all functionally involved in replication of the viral genome (reviewed in [44]). E2, a DNA binding protein, can bind to a motif in the long coding region (LCR) adjacent to the viral origin of replication, thereby recruiting E1, a helicase, compulsory for initiation and elongation of replication of the viral genome [45]. Moreover, E2 is required in dividing basal cells to anchor the episomal genome of the virus to the mitotic chromosomes, thereby ensuring appropriate segregation of the replicated viral genome [46]. Furthermore, E2 acts as a transcriptional regulator of E6 and E7 expression, by binding to the p97 promoter of the virus.

E4 is able to induce cell-cycle arrest in G_2 and could antagonise cell proliferation that would be mediated by E7 in differentiating cells. The E1-E4 fusion protein interacts with cytokeratins to augment the release of new viral particles [47]. E5 contributes to the viral propagation by stimulation of the replication machinery. E5 inhibits the degradation of epidermal growth factor receptor (EGFR). As a consequence, the cell becomes more susceptible for EGF stimulation, which may contribute to a supportive environment for viral replication [48,49]. Due to the capacity of E5 to modulate cell signalling, E5 can aid malignant transformation by E6 and E7, as discussed below [50].

The viral genes *E6* and *E7*, which expression also increases during differentiation, are necessary to induce the DNA replication machinery in otherwise non-dividing cells. E6 and E7 do not hold intrinsic enzymatic activities, but function through direct and indirect interactions with a number of cellular proteins, thereby disturbing their normal functions. Through these interactions, E6 and E7 impede apoptosis and cell cycle control mechanisms of the host cell to allow viral DNA replication in non-dividing differentiating cells (Figure 3). E6 inactivates the tumour suppressor gene p53, through recruitment of E6AP, a cellular ubiquitin ligase, thereby initiating ubiquitin-mediated degradation of p53 and preventing p53-mediated apoptosis upon DNA damage [51-53]. The main function of E7 is to associate with the cell cycle regulator pRb and related pocket proteins, causing disruption of their complex with the transcription factor E2F [54,55]. Released E2F trans-activates cellular genes necessary for the onset of DNA replication [56,57].

Productive infections, in which new viral particles are formed and released, may induce mild histomorphological abnormalities, such as mild dysplasia (CIN1), but do not necessarily reflect a pre-cancerous stage.

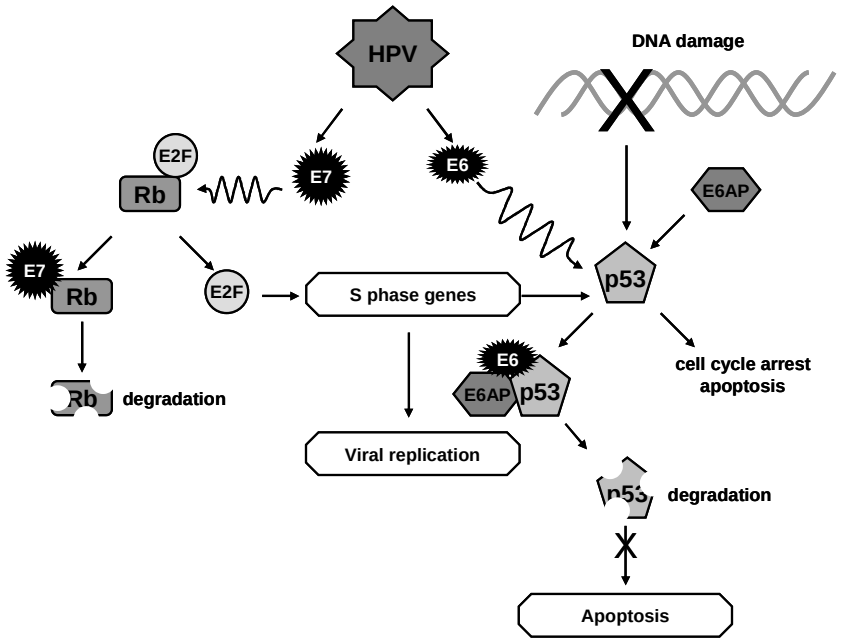


FIGURE 3. Interference of E6 and E7 with cell cycle control and apoptosis mechanisms in differentiated epithelial cells. (Partially adapted from Pearson Education, Inc.).

Transforming hrHPV infections

Contrary to productive infections, transforming infections are associated with high-grade CIN lesions and invasive carcinomas. Transforming infections are characterised by uncontrolled expression of the viral oncoproteins E6 and E7 in the proliferating basal cells of the epithelium. As a consequence of the interactions with the cellular proteins described above, aberrant expression of E6 and E7 in basal dividing cells offers a mechanism of transformation. E6-mediated interference with both p53 function and that of the pro-apoptotic protein Bak [58] prevents cells from undergoing apoptosis. Proteins with a PDZ-binding motif, including Dlg, Scribble, MAGI-1, -2 and -3, MUPP1, CAL and TIP-2/GIPC, can interact directly with E6 *in vitro*. However, the role of these interactions during viral replication and carcinogenesis needs further elucidation [59]. E7 is the second major transforming protein. By its interaction with pRb, it interferes with the control at the G1/S transition of the cell cycle. Inactivation of

pRb by E7 in proliferating cells can be identified by an up-regulation of its upstream inhibitor $p16^{INK4A}$. Since $p16^{INK4A}$ expression is regulated by an pRb-dependent negative feed-back loop, continuous inactivation of pRb by E7 results in strongly increased $p16^{INK4A}$ levels as can be detected in high-grade CIN lesions and cervical carcinomas [60]. In addition to pRb-family members, E7 has been shown to interact with other host cell factors (reviewed by [59,61]). The interference with apoptosis and uncontrolled proliferation, exerted by E6 and E7, is likely to result in a state of genetic instability, enhancing the risk of malignant transformation. However, the exact mechanisms by which E6/E7 expression is deregulated are still not entirely elucidated. A potential mechanism could be inactivation of E2, given that E2 acts as a transcriptional regulator of E6 and E7. Interestingly, upon viral integration the locus of *E2* is often disrupted [62,63]. Yet, recent data propose that integration is not the source but rather a consequence of increasing genomic instability [64]. In addition, the frequency of viral integration in cervical lesions was recently shown to be hrHPV type dependent. Integration of HPV16, 18 and 45 was found considerably more often than integration of HPV31 and 33 [64]. This implies that other mechanisms are responsible for the deregulation of E6/E7 in at least part of the lesions. One such a mechanism may involve DNA methylation of the viral genome, in particularly methylation of the regulatory sequences [65] (see *DNA methylation alterations during cervical carcinogenesis: HPV*).

Concept of hrHPV-induced cervical carcinogenesis

The traditional concept of cervical carcinogenesis proposed that cervical carcinomas progress through a long-lasting sequence of CIN1-CIN2-CIN3 lesions (Figure 4A). In principle, all these lesions can regress, persevere, or progress, although the regression rate lessens with increasing severity. The reverse holds true for perseverance and progression rate.

Current understanding of the role of hrHPV in cervical carcinogenesis has led to novel concept, according to which high-grade CIN lesions may develop fairly

fast following an hrHPV infection without a preceding noticeable CIN1 lesion (reviewed in [15,66]; Figure 4B). In this concept, CIN1 lesions and a subset of lesions scored as CIN2 would simply reflect a productive infection state rather than pre-cancer. Within the group of hrHPV-infected lesions, only lesions harbouring a transforming infection (i.e. subset of CIN2 and CIN3 lesions) have a considerable progression risk if left untreated and should therefore be considered true precancerous lesions (Figure 4B) [67]. Successive development of an invasive carcinoma may take another 10-30 years [68-70]. This is sustained by retrospective analysis of an unethical medical study, conducted in New Zealand between 1965 and 1974, in which CIN3 lesions were intentionally left untreated. Of women whose high-grade disease was initially confirmed and apparently radically treated by a sole biopsy, still 31.3% developed invasive cervical cancer after 30 years. This percentage increased to 50.3% in the subset of women who showed persistent CIN3 lesions within 24 months after the initial biopsy treatment, thus having had inadequate initial management [67].

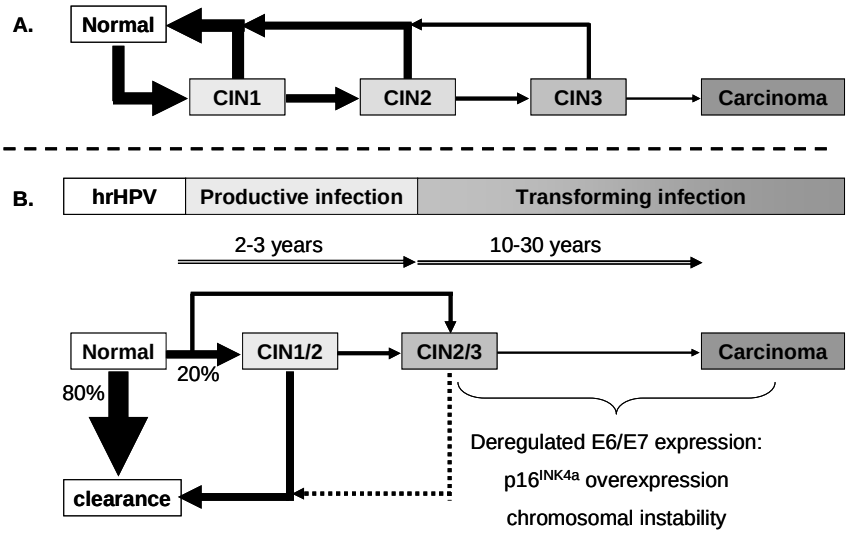


FIGURE 4. (A) Traditional concept of cervical carcinogenesis, based on histological grading. **(B)** Current knowledge about the role of hrHPV is implemented in the classical concept. Adapted from [71].

Contrastingly to SCCs, little is known about the different stages of precursor lesions preceding AdCAs up to adenocarcinoma *in situ* (ACIS). Classification schemes for AdCA precursors prior to ACIS, called cervical intraepithelial glandular neoplasia (CIGN), have been postulated [72], but still remain controversial.

HPV-INDUCED TRANSFORMATION *IN VITRO*

Transformation of primary human keratinocytes in vitro

To increase insight into molecular mechanisms underlying HPV-induced malignant transformation of epithelial cells, *in vitro* model systems have been developed. HPV16 and 18 can induce immortalisation of human epithelial cells of assorted origins, including cervix, foreskin, bronchus, oral cavity, breast and tonsil [73-78]. Two phases can be distinguished within this immortalisation process [79]. First, continued expression of the viral oncogenes *E6* and *E7* leads to deregulation of cell cycle control, resulting in an extended lifespan. Successive acquisition of an immortal phenotype has been recognised as the first phenotypical alteration in the multistep process of malignant transformation mediated by these hrHPVs requiring oncogenic alterations in the host cell. Prolonged culturing of hrHPV-immortalised keratinocytes may lead to additional genetic alterations leading to successively anchorage independence and malignant transformation of these cells [80,81]. This course can be accelerated by introduction of carcinogens or radiation [82-84].

Cell complementation studies have illustrated that for each of the different stages of hrHPV-mediated transformation, as shown in Figure 5, recessive alterations in the host cell genome are necessary [85].

HPV-mediated immortalisation in vitro

To become immortal, a cell must first bypass the barrier of replicative senescence (reviewed in [86]). Normally, the chromosome ends, referred to as telomeres, shorten with each cell division, due to the incapacity of DNA polymerase to entirely replicate the ends of a linear DNA template. This telomere shortening is believed to operate as a mitotic clock, controlling the number of divisions a cell will undergo. At one point in time, telomeres are too short to stabilise the chromosome ends, resulting in the configuration of unstable dicentric chromosomes and the induction of senescence. However, like embryonic cells, germ cells and lymphocytes, many human cancers display increased telomerase activity, thereby preserving their replicative capacity [87]. Telomerase is a ribonucleoprotein complex, able to add 6 base pair repeats (TTAGGG) to telomere ends, thereby preventing telomere shortening and circumventing replicative senescence. The telomerase complex consists of 2 major subunits: a structural RNA component (hTR) and a catalytic subunit (hTERT). The latter possesses reverse transcriptase activity. Whereas hTR is expressed in both normal cells and cancer cells, hTERT expression is largely restricted to cancer cells [88].

hrHPV-mediated transformation *in vitro* is associated with telomerase activity in order to attain an immortal phenotype [79]. Upon over-expression, HPV16 E6 can, either or not in conjunction with c-myc, induce transcription of hTERT [89-91]. E6 can indirectly increase hTERT expression via E6AP-dependent degradation of NFX1-91, a natural repressor of the hTERT promoter which intervenes with the hTERT chromatin structure [92]. A novel level of regulation has been described at the post transcriptional level involving NFX1-123, which, like NFX1-91, is also a splice variant of NFX-1 [93]. Nevertheless, in the context of the full-length viral genome E6 activity alone is insufficient for increased hTERT and telomerase activity. In fact, microcell-mediated chromosome transfer studies have indicated that chromosomes 4q, 6q and 10p are likely to encode proteins or non-coding RNAs that contribute to upregulation of telomerase activity in HPV infected cells

[94-97]. Moreover, hrHPV-induced immortalisation has been associated with a variety of genetic and epigenetic alterations in host cell genes [98-100].

Progression from an immortal to an anchorage independent and tumourigenic phenotype has been linked to the loss of a tumour suppressor gene at chromosome 11 [96,101,102]. Further studies have identified CADM1, a cell adhesion molecule located at 11q23, as a tumour suppressor gene functionally involved in cervical carcinogenesis. Re-expression of CADM1 in SiHa, a cervical cancer cell line, repressed the anchorage-independent and tumourigenic, but not the immortal phenotype of these cells. CADM1 gene silencing by promoter methylation was uncovered to be a common event in the progression from high-grade CIN lesions to cervical cancer [99]. Similar to CADM1, LMX-1A, a homeobox transcription factor located at 1q24, repressed colony formation and invasion *in vitro* of cervical cancer cells upon over-expression, whereas cell proliferation in monolayer cultures was not affected [103]. The acquisition of a tumourigenic phenotype has also been linked to a shift in AP-1 composition, from Fra-1/c-Jun in HPV-immortalized, non-tumourigenic cells to c-Fos/c-Jun heterodimers in tumourigenic cells [104]. A schematic representation of hrHPV-mediated transformation *in vitro* is depicted in Figure 5.

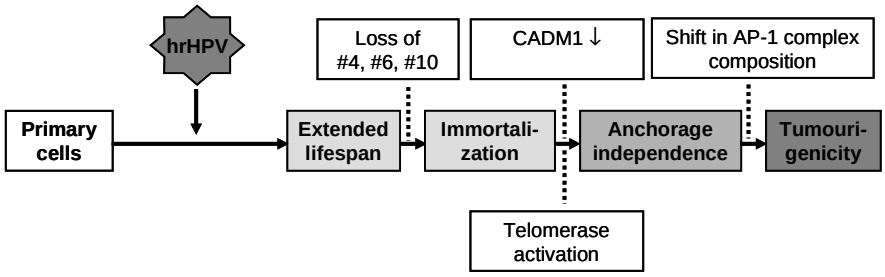


FIGURE 5. Schematic representation of hrHPV-mediated transformation *in vitro*.

HPV TESTING IN CERVICAL CANCER SCREENING

Given that practically all cervical carcinomas are hrHPV-positive and hrHPV is causally involved in the development of cervical cancer, hrHPV testing would be a means to improve existing cervical screening programs. Indeed, large randomised screening trials have shown that testing for hrHPV provides a superior protection against cervical (pre-)cancerous lesions and cervical cancer compared to cytology, and is therefore an attractive primary cervical screening tool [105-107]. Most HPV assays detect the viral DNA by means of either target amplification by PCR or signal amplification following probe hybridisation (i.e. hybrid capture 2 principle). The commercial, FDA-approved (USA) hybrid capture 2 assay (Qiagen) as well as the GP5+/6+ PCR (adapted from the GP5/6 PCR [108]) are presently clinically validated for cervical screening purposes [109].

In addition, hrHPV testing may improve the detection of ACIS and AdCA. These lesions are recurrently overlooked by conventional cervical cytology, probably due to a lower accessibility of the location of the lesion (higher in the endocervical canal compared with CIN lesions and SCCs) [110]. Finally, recent studies have shown that hrHPV testing can also be applied efficiently to cervico-vaginal specimens obtained by self-sampling, for which cytology is not an option [111]. Offering self-sampling to women who do not attend regular screening (e.g. non-responders) resulted in response rates of about 30%, which was significantly higher than what was achieved after a second re-call for conventional cytomorphologic examination [112,113]. Moreover, the number of CIN2+ and CIN3+ lesions detected by hrHPV testing in non-responders was significantly higher than in women responding to the regular screening program. The response rate and the yield of high grade lesions thus support implementation of this method for such women [113].

However, hrHPV testing results in the detection of a substantial number of women with transient hrHPV infections that do not give rise to a clinically meaningful lesion. Consequently, there is a need to discover markers that will facilitate a better risk stratification of hrHPV-positive women, thereby reducing the number of redundant follow-up scrapings and visits to the gynaecologist. Presently, cytology is the best available triage tool for hrHPV-positive women. Still, this method does not present a solution for women with normal cytology, which form the largest group of hrHPV-positive women. Additional candidate biomarkers can be HPV-based, for instance viral load assessment [15], HPV E6/E7 mRNA detection [114,115] or viral typing. Recent studies showed that risk and clearance rates in women with normal or mildly abnormal cytomorphology were dependent on the HPV type present. HPV16, and to a lesser extent HPV18, 31, and 33, showed significantly decreased clearance rates, pleading for closer surveillance of women infected with these types [105,116]. Alternatively, host cell biomarkers can be of value, such as over-expression of $p16^{INK4a}$, which, as discussed before, reflects transforming hrHPV infections [117-119]. However, as discussed below, $p16^{INK4a}$ immunostaining is still a subjective tool and reflects a rather early step in cervical carcinogenesis, indicating the need for additional, preferentially objective, molecular markers that can identify the true cervical cancer precursor lesions.

MECHANISMS OF (EPI)GENETIC ALTERATIONS

As discussed above, cervical cancer is a long term process resulting from the accumulation of genetic and epigenetic alterations in the host's onco- and tumour suppressor genes.

Activation of oncogenes can be accomplished by activating mutations, initiating altered protein amounts or protein products, with c-myc, Ras and EGFR being

well-examined examples. Furthermore, numerical and structural chromosomal alterations are frequent in solid tumours and can initiate altered expression of oncogenes. Gains of chromosomal regions can give rise to increased expression of oncogenes located at the gained regions.

Inactivation of tumour suppressor genes can also be achieved through a diversity of mechanisms. For example, inactivating mutations have been described for the tumour suppressor genes pRb, p53, APC and BRCA-1 and -2. Next to mutations, deletions may also contribute to decreased tumour suppressor gene activity.

Alternatively, tumour suppressor genes can become inactivated by epigenetic modifications. Those epigenetic modifications do not affect the DNA sequence itself, but rather the accessibility of the DNA for transcription factors, by this means influencing gene expression. This is achieved by altering either bases in the DNA sequence or the histone proteins, around which the DNA is enfolded. These modifications include methylation, ubiquitination, phosphorylation and acetylation of histones, as well as methylation of CpG dinucleotides in the DNA. The latter involves the covalent binding of a methyl-group (CH3) at the carbon-5 position of cytosine located 5'of a guanine, to retrieve a 5-methylcytosine. The physiological function of DNA methylation may be an overall genetic stability and maintenance of chromosomal integrity and to facilitate organisation of the genome into active and inactive regions with respect to gene transcription [120-122]. Genes with CpG islands in the promoter regions are unmethylated in normal tissues. Exemptions are inactivated genes on the female X-chromosome and inactivated alleles of selected imprinted genes on autosomal chromosomes. For the past decade, abnormal patterns of DNA methylation, e.g. methylation of CpG islands in the DNA sequence of the promoter region of tumour suppressor genes, have been recognised as molecular changes in neoplasia [123]. In comparison to normal cells, cancer cells display global hypomethylation, whereas simultaneously specific hypermethylation of certain (normally unmethylated) gene promoters is observed (Figure 6).

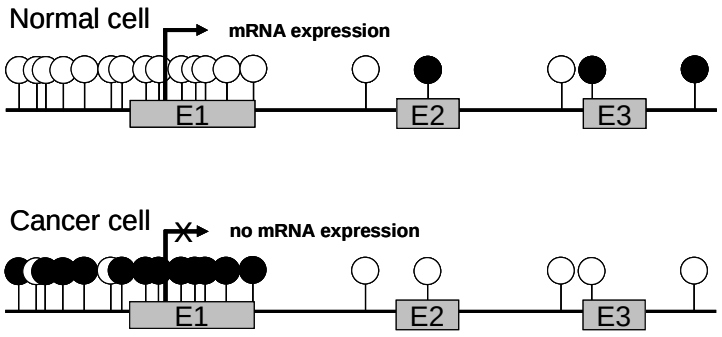


FIGURE 6. Schematic representation of promoter methylation in a normal cell and a cancer cell. White circles represent unmethylated CpGs, black circles depict methylated CpGs. In normal cells, transcription factors bind the gene promoter, enabling mRNA expression. In cancer cells, transcription factors cannot bind to the methylated gene promoters, thereby inhibiting mRNA expression.

Recently, the discovery of a class of small non-coding RNAs, so called microRNAs (miRNAs), has gained notice in oncology research. MiRNAs are regulatory RNAs of 20–30 nucleotides in length, that can bind to the 3′ untranslated regions (3′UTR) of target mRNAs, resulting in the degradation or inhibition of mRNA translation (reviewed in [124]). It is the function of the target mRNA that determines a miRNA acting either tumour suppressive (if directed against proto-oncogene transcripts) or oncogenic (if directed against tumour suppressor gene transcripts). Currently, more than 800 human miRNAs have been described and the number of genes known to be regulated by miRNAs, is growing rapidly. Like protein-coding genes, DNA sequences encoding miRNAs were found to be a target of aberrant DNA methylation [125,126], explaining in part how miRNAs may be down-regulated (through DNA methylation) in cancer.

HOST-CELL ALTERATIONS ASSOCIATED WITH HPV-INDUCED CARCINOGENESIS

Both *in vitro* systems and cervical lesions have been extensively studied by a diversity of molecular techniques to determine the (epi)genetic events occurring during cervical carcinogenesis. Numerous alterations have been found, a summary of which will be given in this section. It remains a demanding task to determine which of these alterations are essentially required for cervical cancer development and which just reflect the general genetic instability induced by HPV. We hypothesise that particularly the alterations being functionally involved in malignant transformation will yield valuable markers for the management of hrHPV-positive women.

The onset of a transforming infection is characterised by deregulated E6/E7 expression in proliferating cells, which leads to uncontrolled cell proliferation, primarily resulting from increased E2F activity. This phenomenon is characterised by an increased expression of proliferation markers like PCNA, Ki-67, minichromosome maintenance proteins (MCMs), Cyclin E and p21 (reviewed by Baldwin *et al.* [127]).

In addition, elevated levels of $p16^{INK4A}$ expression, resulting from E7-mediated interference with an Rb-dependent negative feed-back loop regulating $p16^{INK4A}$ expression, has been linked to CIN lesions and carcinomas harbouring a transforming hrHPV-infection [60].

Following a transforming infection immortalisation may occur, which is characterised by activation of telomerase. Telomerase activity and elevated hTERT mRNA expression has been shown in nearly all cervical carcinomas and almost half of CIN3 lesions, whereas normal cervix, CIN1 and CIN2 lesions were devoid of detectable telomerase activity [128].

However, telomerase activity and elevated hTERT expression cannot be reliably detected in cervical scrapings [129-131]. In a study by de Wilde *et al*., using an *in vitro* model of telomerase-positive and -negative HPV16-transformed cells, a number of surrogate markers for deregulated hTERT mRNA expression and telomerase activation were defined, including AQ3 down-regulation and MGP up-regulation [132].

Genome-wide micro-array-based comparative genomic hybridisation (array CGH) studies on cervical carcinomas have identified a number of common chromosomal alterations, including gains at 1q, 3q and 20q and losses at 2q, 3p, 4q, 6q, 11q and 13q [133-147]. Interestingly, comparison of micro-array CGH profiles of high-grade CIN lesions with cervical SCCs revealed that a subset of high-grade CIN lesions has CGH profiles that were more closely related to cancers than others. This shows that high-grade CIN lesions can be sub-categorised with respect to their chromosomal profiles, which may enable the distinction of CIN lesions with short-term invasive potential [146].

Presently, also multiple micro-array expression studies have been conducted on cervical carcinomas, that resulted in extensive lists of genes that are either over-expressed or under-expressed in cervical cancers [148-154]. A number of differentially expressed genes have been linked to a chromosomal alteration, including *DTX3L*, *PIK3R4*, *ATP2C1* and *SLC25A36*, all located at 3q21 [155].

Next to protein encoding genes also altered expression of an increasing number of microRNAs have been described in cervical cancer cell lines and carcinomas, such as miR-126, miR-143, and miR-145 being down-regulated and miR-15b, miR-16, miR-146a, and miR-155 showing up-regulation [126,156-160]. Future research into the area of miRNAs will most likely elucidate more key players in cervical cancer and will thus add to our understanding of cervical carcinogenesis.

DNA methylation alterations during cervical carcinogenesis: HPV

HPV itself can become subject to DNA methylation during cervical carcinogenesis. *De novo* methylation of HPV DNA was understood as being a host defence mechanism for silencing viral replication and transcription that is utilized by the virus to preserve a long-latency infection [65,161-163]. HPV16 and HPV18 DNA methylomes (e.g. methylation pattern of every CpG of the DNA) from pre-immortal keratinocytes were found to be almost completely unmethylated, while the immortal descendant cells and CaSki and HeLa cervical cancer cell lines featured densely methylated viral genomes [65].

Recent studies on cervical samples have shown that, in general, increased viral DNA methylation is associated with the severity of cervical disease [65,164-170]. In some studies, the LCR and E6 sequences of HPV16 and HPV18 were commonly found to be unmethylated independent of the stage of neoplastic progression, whereas the L1 region was densely methylated in cancers [164,165,168-170]. However, in other studies, methylation of the LCR was observed in primary cervical carcinomas, especially at the E2-binding sites [65,166,167]. *In vitro* it has been shown that DNA methylation of the E2-binding site inhibits the binding of E2 [171], and that this methylation is related to reactivation of E6 and E7 viral proteins [65]. Nevertheless, E6 and E7 expression levels are still kept into pace by methylation in case of multiple HPV copies, such as in CaSki cells. In these cells it was shown that active viral RNA transcription occurs at only a single-copy or low copy-number site on a derivative of chromosome 14, whereas all other loci, harbouring up to 600 viral copies, were inactive. Transcription from the silent (i.e. methylated) viral DNA copies was activated upon growth in the presence of 5-azacytidine [172].

Although in general, increased HPV DNA methylation is correlated with progression to malignancy, results on the specific CpGs being targeted by methylation and being predictive for progression are not consistent and would require a more in depth analysis. One complication seems to be the presence of multiple viral DNA copies in a lesion, of which only a minority is expected to be actively transcribed [172].

DNA methylation alterations during cervical carcinogenesis: host cell genes

A rapidly growing number of studies have described aberrant methylation of established or candidate tumour suppressor genes in cervical carcinoma biopsies, as summarised in Table 1. Those include genes involved in apoptosis, WNT-signalling, Ras-signalling and in tumour invasion and metastasis.

Most studies used either bisulfite sequencing or methylation-specific PCR (MSP). Both methods are based on conversion of unmethylated Cytosines to Uracils by bisulfite treatment. Whereas for bisulfite sequencing primers flanking methylated CpG sites are used, MSP is based on the specific amplification of methylated DNA using primers targeting methylated CpGs. As a control, corresponding unmethylated DNA or a house-keeping gene is PCR amplified. The variation amongst the different studies, as for example DAPK1 methylation varies from 0% to 100% and APC methylation varies from 6 to 94%, may at least in part be explained by the use of different techniques that have different analytic sensitivities, analysis of different promoter regions within the same gene in combination with heterogeneity in methylation patterns, or to biological variation between populations.

A number of genes were recurrently found to be methylated in cervical carcinomas (i.e. in ≥2 studies), including CALCA, CCNA, CDH1, CADM1 and DAPK1. One of the genes studied in both cancers and pre-malignant lesions is CADM1, which was found to be functionally involved in cervical carcinogenesis (see section *HPV-mediated immortalisation in vitro*). Using bisulfite sequencing analysis, CADM1 promoter methylation was detected in 35% of high-grade CIN lesions and in 58% of cervical SCCs [99]. This high frequency of CADM1 methylation in cervical carcinomas and precursor lesions was confirmed by others [173-175].

Gene	ICCs	References
APAF1	14.1%	176
APC	11.0-93.8%	98,175,177-182
BLU	70.9%	183
BRCA1	6.1%	178
CADM1	41.7-65.2%	98,99,174,175
CALCA	63.3-95.0%	175,184
CASP8	3.5-3.8%	176,185
CAV1	5.6%	186
CCNA1	51.7-93.3%	175,187,188
CDH1	13.3-88.8%	178,179,181,189-192
CDH13	40.0-87.5%	98,192
CDKN2A	6.7-8.5%	178,192
CDKN2B	8.3%	98
CHFR	37.5%	98
COX2	14.6%	181,191
DAPK1	20.0-100%	98,175,176,178-182,189-193
DAZL	100%	188
DcR1	100%	194
DcR2	18.0%	194
Dkk3	31.4%	195
DLC1	87.5%	196
E-cadherin	28.3-80.5%	180,181,197-199
ESR1	25.0-30.0%	98,175,182
FANCF	29.7%	200
FAS	64.7%	176
FHIT	11.0-100%	178,181,182,191,198,201-204
GSTP1	0-88.3%	181,182,202
HIC1	18.3-45.3%	178,180
HIN1	18.2%	205
HLTF	3.7-16.3%	179,181,191
HS3ST2	93.3%	190
hTERT	0-58.3%	184,192,206
HTRA3	11.1%	188

Gene	ICCs	References *(Continued)*
HS3ST2	93.3%	190
ITGA9	23.6%	207
LHFPL4	66.7%	192
LMX1A	89.9%	208
MGMT	4.9-91.7%	98,178,180-182,191,193,202
MLH1	2.2-12.7%	179,181,191,202,207
MYOD1	25.6%	184
NKX6-1	80.4%	208
NNAT	100%	188
NOL4	53.3%	192
NPTX1	50.0%	188
ONECUT1	20.4%	208
p14	4.9-6.1%	181,191
$p16^{INK4A}$	3.1-59.1%	179-182,189,191,193,197,202,209-211
p73	0-50.0%	98,178,212
PAX1	94.4%	208
PCDH10	85.7%	213
PGR	77.8%	184
POU2F3	37.8%	214
PRDM5	40.5%	215
PTEN	58.1%	216
RARβ	12.5-85.0%	98,175,178,182,202,203,217
RASSF1A	0-24.0%	98,177-179,181,183,191,203,218-221
RBSP3	24.5%	207
Reprimo	18.4%	222
RIZ1	37.5%	223
ROBO1	46.2%	224
ROBO3	35.6%	224
RUNX3	2.4-2.5%	181,191,225
SFRP1	52.2%	226
SFRP2	82.6%	226
SFRP4	65.2%	226

Gene	ICCs	References	*(Continued)*
SFRP5	73.9%	226	
SLIT1	52.9%	224	
SLIT2	58.0-63.9%	224,227	
SLIT3	49.2%	224	
SOCS2	53.3%	192	
SOCS3	26.7%	190	
SOX1	81.5%	208	
SPARC	95.0%	175	
SST	77.8%	188	
STAC	19.1%	207	
SYCP3	100%	188	
TFPI2	58.3%	175	
THBS1	34.8-37.8%	179,181,191	
TIMP2	47.2%	228	
TIMP3	1.2-40.0%	98,178,179,181,184,189,191,192	
TMS1/ASC	6.2%	185	
TNFRSF10C	48.9%	190	
TRAIL R1	68.2%	176	
WT1	77.8%	208	
ZFP43	100%	188	

TABLE 1. Frequency of tumour suppressor gene methylation in cervical carcinomas. If in a study both SCCs and AdCAs were analyzed, the depicted percentages are a reflection of the average of the methylation frequencies of both groups.

It should, however, be noted that most studies examining molecular mechanisms, including epigenetic events, underlying cervical carcinogenesis have focused on cervical SCC and their squamous precursor lesions (e.g. CIN lesions). Recent studies on both cervical SCCs and AdCAs indicate however that not only the hrHPV type distribution [229] and genetic signature [145] differ between both histotypes, but that also the epigenetic alterations may be dissimilar. For example, genes like DAPK1, BLU, CCNA and PCDH10 show higher methylation frequencies in cervical SCCs compared to AdCA [98,175,176,178-

[180,183,189,190,193,213]. On the other hand, APC, BRCA1, HIC1, RASSF1A, TIMP3, p73 and SOCS3 are more commonly methylated in cervical AdCAs compared with SCC [98,177-180,183,184,189,190,220,221]. The methylation alterations specifically found in AdCAs may in the future be useful to identify women at increased risk for developing ACIS or AdCAs and who therefore will require endocervical curettage.

DNA methylation alterations detected in cervical cancers are of particular interest, as recent studies indicate that DNA methylation can be easily detected in cervical scrapings, using MSP or quantitative MSP (qMSP). qMSP is based on the same principle as MSP, but uses real-time PCR amplification often in combination with a probe specific for methylated DNA.

Interestingly, (q)MSP analysis of cervical scrapings was found to closely reflect methylation in the underlying epithelium [230,231]. Nevertheless, not all methylation results obtained from tissue samples, as summarized in Table 1, can be directly extrapolated to cervical scrapings. Cervical tissue samples often contain substantial amounts of non-epithelial (stromal) cells, whereas cervical scrapings are enriched with superficial epithelial cells, each of which may display distinct levels of background methylation.

Present data on methylated gene promoters investigated in cervical scrapings and liquid-based cytology are recapitulated in Table 2.

In studies on scrapings of cervical cancer patients and controls, genes like CCN1A, SPARC, hTERT and TIMP3 were shown to be highly discriminative between both groups [187,232,233]. Besides these individual markers, analyses of gene panels on cervical scrapings have shown high sensitivities and specificities for cervical cancer. For example, the combination of CALCA, DAPK1, ESR1 and APC qMSP analyses on cervical scrapings showed a sensitivity of 89% for cervical cancer with a fixed specificity of 100%, calculated for cancer-free subjects visiting an outpatient clinic [234]. Yet, the diagnostic value of these methylation events in a screening or referral setting remains to be determined.

Gene	N/LSIL	HSIL	ICC	References
APC	35.1%	34.2%	31.8%	230
	39.0%	-	54.2%	231
	0%	-	17.9%	234
	88.2%	83.3%	60.0%	175
	70.8%	66.7%	-	235
ASC	3.0%	0%	6.7%	230
C13ORF18	2.1%	37.1%	70.1%	236
CADM1	5.0%	25.6%	-	237
	0%	45.5%	-	173
	0%	-	54.5%	99
	64.7%	61.1%	75.0%	175
	100%	100%	-	235
CALCA	0%	-	67.8%	234
	100%	100%	100%	175
CCNA1	17.6	55.6%	75.0%	175
	4.2%	37.1%	70.1%	236
CCND2	2.8%	0%	7.4%	230
CDH1	13.0%	16.7%	19.2%	230
	23.1%	38.7%	90.9%	233
	0%	7.5%	4.3%	238
	79.2%	83.3%	-	235
CDH13	-	8.3%	-	192
	4.0%	4.0%	9.5%	239
	17.0%	15.6%	46.1%	230
	7.7%	12.9%	81.8%	233
	0%	8.3%	-	235
CDKN2A	2.9%	2.2%	4.8%	230
	25.0%	36.4%	-	173
CDKN2B	2.4%	2.2%	3.7%	230
DAPK1	3.3%	25.6%	-	237
	-	33.3%	-	192
	4.0%	28.0%	49.2%	239
	30.0%	-	-	240
	0%	-	46.4%	234
	3.4%	34.8%	55.0%	230
	0%	63.6%	-	173
	4.9%	-	72.9%	231
	7.7%	22.6%	81.8%	233
	64.7%	27.8%	90.0%	175
	34.2%	73.1%	72.5%	238
	12.5%	16.7%	-	235

Gene	N/LSIL	HSIL	ICC	References *(Continued)*
ESR1	0%	-	32.1%	234
	11.8%	11.1%	50.0%	175
FHIT	3.4%	2.2%	7.8%	230
	0%	0%	-	235
GSTP1	1.2%	2.2%	0%	230
	0%	-	2.1%	231
	0%	6.5%	18.2%	233
	0%	0%	2.9%	238
	4.2%	0%	-	235
HIC1	0%	-	3.6%	234
	65.1%	66.6%	70.9%	230
	17.6%	27.3%	-	173
	34.2%	59.7%	59.4%	238
	100%	100%	-	235
HIN1	13.7%	26.9%	33.3%	238
HOXA9	100%	100%	-	235
HOXA10	68.4%	81.0%	-	235
HOXA11	94.7%	100%	-	235
HOXC9	60.5%	61.9%	-	235
HOXD9	100%	100%	-	235
HSPA2	0%	3.2%	72.7%	233
hTERT	-	16.7%	-	192
	51.9%	77.8%	-	241
	0%	0%	81.8%	233
LHFPL4	-	25.0%	-	192
LMX1A	10%	16%	36.4%	208
	12.5%	25.0%	-	235
MGMT	2.2%	0%	11.1%	230
	7.1%	0%	-	173
	14.6%	-	10.4%	231
	2.7%	11.9%	26.1%	238
MLH1	0%	-	10.7%	234
	0%	2.2%	1.1%	230
	0%	3.2%	36.4%	233
	7.1%	18.2%	-	173
	0%	1.5%	0%	238
MT1G	4.8%	-	54.5%	232
	18.4%	19.0%	-	235
NKX6-1	33.3%	55.1%	63.6%	208
	78.9%	76.2%	-	235

Gene	N/LSIL	HSIL	ICC	References *(Continued)*
NMES1	0%	-	36.4%	232
NOL4	-	41.7%	-	192
ONECUT1	3.3%	7.4%	13.6%	208
	100%	91.7%	-	235
p14	7.1%	18.2%	-	173
p16^{INK4A}	20.0%	55.6%	-	242
	9.6%	11.9%	13.0%	238
p73	14.3%	18.2%	-	173
PAX1	1.2%	42.1%	86.4%	208
	2.6%	28.6%	-	235
PCDH10	0%	21.8%	71.0%	213
	8.2%	46.0%	90.9%	243
PRDM2	1.8%	0%	2.6%	230
RARβ	4.0%	4.0%	39.7%	239
	0%	-	17.9%	234
	2.6%	9.1%	38.2%	230
	0%	9.1%	-	173
	41.2%	55.6%	95.0%	175
	9.6%	46.3%	53.6%	238
	94.7%	100%	-	235
RASSF1A	0%	-	7.1%	234
	2.8%	0%	1.1%	230
	3.6%	9.1%	-	173
	7.7%	0%	45.5%	233
	8.2%	25.4%	30.4%	238
ROBO1	4.0%	8.3%	-	224
ROBO3	0%	10.3%	-	224
RRAD	42.9%	-	68.1%	232
SFN	92.6%	97.0%	98.6%	230
SFRP1	4.8%	-	59.1%	232
	1.1%	8.2%	33.9%	244
SFRP2	10.0%	16.3%	80.7%	244
SFRP4	2.2%	36.7%	67.9%	244
SFRP5	8.9%	4.1%	10.1%	244
SHP1	13.7%	32.8%	33.3%	238
SLIT1	0%	10.3%	-	224
SLIT2	1.3%	25.0%	-	224
SLIT3	2.0%	2.4%	-	224
SOCS1	0%	7.1%	54.5%	233

Gene	N/LSIL	HSIL	ICC	References *(Continued)*
SOCS2	23.1% 4.2%	45.2% 0%	63.6% -	233 235
SOX1	3.3% 76.3%	9.3% 95.2%	68.2% -	208 235
SPARC	16.7% 4.8% 88.2%	48.7% - 94.4%	- 90.9% 100%	237 232 175
SYK	5.0%	4.0%	15.1%	230
TFPI2	3.3% 38.1% 24.5%	23.1% - 38.9%	- 81.8% 80.0%	237 232 175
TIMP3	- 0% 0% 8.3%	8.3% - 16.1% 8.3%	- 21.4% 100% -	192 234 233 235
TWIST1	4.0% 0% 9.6% 13.2%	4.0% 13.6% 40.3% 14.3%	31.7% 42.9% 34.8% -	239 230 238 235
VHL	0.8%	2.3%	1.6%	230
WT1	15.6% 21.1%	42.1% 38.1%	77.3% -	208 235

TABLE 2. Frequency of tumour suppressor gene methylation in cervical scrapings, as determined in individual studies; N=normal cytology; LSIL= low-grade squamous intraepithelial lesion; HSIL= high-grade squamous intraepithelial lesion; ICC=invasive cervical carcinoma; (-) = sample group was not tested in the presented study.

OUTLINE OF THIS THESIS

It is apparent that hrHPV is causally related to the development of cervical cancer and comprehensive knowledge about the mechanisms by which hrHPV initiates transformation of epithelial cells is present. The course of malignant transformation, however, is still not entirely understood. (Epi)genetic alterations in the host cell genome are crucial for malignant transformation and more insight into these alterations may add to a better understanding of HPV-

mediated carcinogenesis. Furthermore, insight into biologically relevant alterations in the host cell genome may facilitate better risk stratification of hrHPV-positive women with respect to the development of cervical cancer.

As summarised earlier, previous studies in our laboratory have yet indicated that HPV-induced carcinogenesis is accompanied by a consecutive number of critical phenotypical alterations. These include a.o. the acquisition of immortality by activation of telomerase, resulting from deregulated hTERT expression, and the anchorage-independent and tumourigenic phenotype being suppressed by CADM1 over-expression. Next to these *in vitro* findings, a number of expression alterations have been identified in cervical carcinomas, with down-regulation of MAL being one of the most prominent findings.

In this thesis we aimed to gain further insight in epigenetic events associated with these specific alterations during cervical cancer development. Not only did we determine their functional relevance but also their potential clinical value as biomarker for early cervical cancer detection. Towards accomplishment of this aim, the following questions were raised:

Chapter 2: *Could deregulated hTERT expression during HPV-induced carcinogenesis result from aberrant DNA methylation of its regulatory sequences?* With the help of luciferase reporter assays containing diverse hTERT regulatory regions, we showed the existence and position of specific repressive sequences in the hTERT promoter. By successive bisulfite sequencing of those regions, we demonstrated that methylation thereof is associated with deregulated hTERT transcription in HPV-transformed cells. Upon analysis of cervical biopsies we observed a gradual increase in methylation of the hTERT regulatory regions with the severity of cervical disease. This indicates that DNA methylation at these regions may provide a biomarker for the early detection of cervical cancer.

Chapter 3: *How does CADM1 promoter methylation relate to in vitro growth characteristics and gene silencing in vivo?*
Methylation-specific PCRs targeting three regions within the CADM1 promoter revealed that density of methylation was associated with the degree of anchorage-independent growth and CADM1 gene silencing *in vitro*. In cervical squamous lesions, methylation frequency and density increased with severity of disease. Dense methylation (defined as ≥2 methylated regions) increased from 5% in normal cervical samples to 30% in CIN3 lesions and 83% in squamous cell carcinomas (SCCs) and was significantly associated with decreased CADM1 protein expression. The frequency of dense methylation was significantly higher in ≥CIN3 compared with ≤CIN1, as well as in SCCs compared to adenocarcinomas.

Although CADM1 methylation was identified as an attractive disease marker for hrHPV-positive women, its sensitivity for CIN3 lesions and AdCA was suboptimal. In search of an additional candidate gene for methylation analysis, we focused on the *MAL* gene, which had recently been identified as the most down-regulated gene in cervical carcinomas and embedded in a CpG island [155].
In **Chapter 4** we raised the following question: *Does MAL gene silencing result from promoter methylation and is MAL gene silencing, like CADM1 gene silencing, functionally involved in cervical carcinogenesis?*
MAL mRNA was (nearly) undetectable in all HPV-immortalised and cervical cancer cells, but could be up-regulated upon methylation inhibition. MAL promoter methylation at two promoter regions (M1 and M2) was detected in all HPV-immortalised cells and cancer cells. Ectopic expression of MAL in SiHa cells suppressed proliferation, migration, and anchorage-independent growth. Furthermore, MAL promoter methylation increased with the severity of the cervical lesions. Moreover, detection of MAL promoter methylation in cervical scrapings was predictive for underlying high-grade lesions. Both in biopsies and in scrapings, MAL promoter methylation was significantly correlated with reduced mRNA expression.

Since MAL silencing was found to occur prior to CADM1 gene silencing in the process of HPV-induced transformation, we hypothesised that methylation of both genes may be at least in part complementary to each other in terms of CIN3 detection. Therefore we questioned in **Chapter 5:** *Does a panel of CADM1 and MAL methylation markers better enable risk stratification of hrHPV-positive women than those methylation markers individually?*

Methylation analysis was performed by qMSP for two regions in both the CADM1 and the MAL promoter on cervical tissue specimens representing the full spectrum of cervical (pre)malignant disease. This resulted in a ≥95% positivity rate for CIN3, SCC and AdCA, while 0% of normal cervices and only 23% of CIN1 lesions were positive. Hereafter, the marker combination with the highest sensitivity and specificity was determined in a subsequent analysis of different methylation marker combinations on hrHPV-positive cervical scrapings. The results indicated that a combined analysis of CADM1 M18 and MAL M1 methylation could detect 90% of underlying high-grade CIN lesions, while only 13% of hrHPV-positive women without high-grade disease were detected.

Ultimately, the performance of this CADM1 M18 / MAL M1 methylation panel following hrHPV testing was validated in a prospective study on women visiting the outpatient colposcopy clinic. The marker panel reached a sensitivity of 70% for ≥CIN3 or cervical cancer. When low-volume lesions were excluded, a sensitivity of 85% could be achieved. Conclusively, these data show that hrHPV testing combined with a panel of CADM1- and MAL-based methylation markers shows a high sensitivity for ≥CIN3 lesions which may provide a new molecular triage marker for hrHPV-positive women. Since MAL silencing by methylation was found to be highly common in both cervical SCCs and AdCAs, we questioned whether also AdCAs arising at other sites in the body may show MAL promoter methylation and gene silencing.

In **Chapter 6** we questioned: *Does MAL promoter methylation also occur in gastric cancer and does it also affect MAL mRNA expression?*
The prevalence of MAL promoter methylation and the association with mRNA expression in gastric cancers were analysed and the methylation status was correlated to clinicopathological data. MAL promoter methylation at two promoter regions (M1 and M2) occurred in 71% and 80% of the gastric cancers, respectively, but not in normal gastric mucosa tissue. Methylation of M2, but not M1, was correlated with significantly better disease-free survival and with down-regulation of MAL mRNA expression. These results indicate that MAL has a putative tumour-suppressor gene function in gastric cancer as well.

Data collected in these studies enabled further updating of the current concept of HPV-mediated carcinogenesis, which is presented in **Chapter 7**.

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