Innovative molecular markers for diagnosis and prognosis in cervical neoplasia

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Chapter 1

General introduction
CHAPTER 1

General introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide. There are 530,000 new cases per year with a mortality of 275,000 per year \(^1\). The majority of cervical cancer occurs in developing countries without a cervical cancer screening program. In the Netherlands around 700 new cases per year are diagnosed, with a corresponding 200 deaths \(^2\).

Cervical cancer is preceded by a premalignant phase: Cervical Intraepithelial Neoplasia (CIN). There are three stages, CIN1, 2 and 3 and while CIN1 regresses in most cases, 20-45% of the CIN2/3 lesions will progress to cancer if left untreated \(^3\). It is estimated that the progression from CIN to cervical cancer generally takes 10-15 years \(^4\). Figure 1 shows the gradual progression of cervical carcinogenesis with the cytological (Pap) classification that is used for screening and histological (CIN) classification that is used for diagnosis. Low-grade squamous intra-epithelial lesions (LSIL) include CIN1 and high-grade squamous intra-epithelial lesions (HSIL) include CIN2/3.

![Figure 1](http://www.sh.tsuhs.edu)

Figure 1. Schematic presentation of the morphological alterations in cervical carcinogenesis with the histological CIN classification and cytological Pap classification (adapted from http://www.sh.tsuhs.edu).

Human papillomavirus

Persistent infection with high-risk human papillomavirus (hrHPV) has been causally related to the development of cervical cancer \(^5\). hrHPV DNA has been detected in 99.7% of all squamous cervical cancers and in 94-100% of the cervical adenocarcinomas \(^5\).
Papillomaviruses are small, double-stranded DNA viruses. The early proteins E6 and E7 are the primary HPV oncoproteins. E6 degrades tumor suppressor protein p53, thereby blocking apoptosis and E7 binds to the retinoblastoma tumor suppressor protein (pRB) and abrogates cell-cycle arrest. Over 170 different types of HPV have been identified of which about 40 are known to infect the genital mucosa. There are 12 hrHPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) that are associated with cervical carcinogenesis and 6 HPV types classified as probable high-risk (HPV26, 53, 66, 68, 73 and 82). HPV 16 and 18 cause approximately 70% of all cervical cancer cases. Although 80% of all sexually active women will be infected with an HPV infection during their lifetime, most HPV infections are transient and most women will clear the HPV infection within 1-2 years after exposure. Only persistent hrHPV infection can attribute to neoplastic progression of cells.

Screening in the Netherlands

In the Netherlands a population-based cervical cancer screening program exists since 1988. Women in the age group 30-60 years are invited every 5 years. The introduction of this national screening program reduced the incidence and mortality of cervical cancer by 40%-50%. The most widely used cervical cancer screening test is based on cytological examination of exfoliated cells derived from the transformation zone (Pap test). For a conventional Pap test, the cervix is scraped with a brush, stained and cytologically evaluated, for which the Pap/CISOE-A or the Bethesda classification system is used (Table 1). This cytomorphological classification system is based on screening and is associated with the underlying histology of the lesion, that is used as the reference standard for diagnosis (Table 1).

<table>
<thead>
<tr>
<th>Cytological classification (used for screening)</th>
<th>Histological classification (used for diagnosis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papanicolaou</td>
<td>Bethesda system</td>
</tr>
<tr>
<td>Pap1</td>
<td>Normal</td>
</tr>
<tr>
<td>Pap2</td>
<td>ASC-US</td>
</tr>
<tr>
<td>Pap3a1</td>
<td>Low-grade SIL</td>
</tr>
<tr>
<td>Pap3a2</td>
<td>High-grade SIL</td>
</tr>
<tr>
<td>Pap3b</td>
<td>High-grade SIL</td>
</tr>
<tr>
<td>Pap4</td>
<td>High-grade SIL</td>
</tr>
<tr>
<td>Pap5</td>
<td>Invasive carcinoma</td>
</tr>
</tbody>
</table>

SIL: Squamous Intraepithelial Lesion
The screening test that is used for primary cervical cancer screening should fulfill certain requirements. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are terms used to evaluate the performance of a screening test. The sensitivity of a clinical test refers to the ability of the test to correctly identify those patients with disease (e.g. the percentage of people with disease who are correctly identified as having the condition). The specificity of a clinical test refers to the ability of the test to correctly identify those patients without the disease (e.g. the percentage of healthy people who are correctly identified as not having the condition). Predictive values of the test depend on the prevalence of disease in the population. The positive predictive value (PPV) is the proportion of people with a positive test result who actually have the disease. The negative predictive value (NPV) is the proportion of people with a negative test result who do not have the disease.

Primary population-based screening by cytological assessment of cervical scrapings shows high specificity (~95%). However, sensitivity for detecting CIN2 or higher (CIN2+) lesions is rather low (~55%). Cytology testing is also characterized by low reproducibility, because of the subjective nature of the test. For hrHPV testing the sensitivity for detecting CIN2+ lesions is much higher (~92%). However, specificity of the hrHPV test, especially in younger women, is around 6% lower than with cytology due to a substantial number of women with transient hrHPV infections that do not give rise to clinically meaningful lesions. Because of the improved sensitivity of hrHPV testing, the Dutch Ministry of Health has recently decided to change the screening program in the Netherlands. Starting from 2016 all women in the age group of 30-60 years will be screened with primary hrHPV testing.

At present different hrHPV-tests exist; there are 4 FDA approved hrHPV tests available (Hybrid Capture 2, Cervista HPV HR assay, COBAS 4800, and the Aptima® HPV assay). Many new hrHPV tests have been developed and to assure high quality of these new hrHPV tests, they should fulfill performance standards as formulated in the international guidelines for HPV testing by Meijer et al.

Triage testing of hrHPV positive women

To prevent unnecessary referral to gynecologists a triage test for hrHPV positive women is needed. The triage test that is now mostly advocated is cytology-based testing, with a sensitivity for CIN2+ between 48%-66% and specificity between 81%-99%. However, because cytology-based testing is prone to subjectivity, more women may be considered cytomorphological abnormal (≥ASCUS) when they are known to be hrHPV-positive. Thereby specificity of this triage test will probably decrease. Other options for triage testing are HPV16/18 genotyping, p16INK4a immunohistochemistry and DNA methylation markers.
**Non-responders**

Apart from the efficacy of the screening test, low participation rate is another aspect in population-based screening programs that could be improved. Around 35% of the women in the Netherlands do not respond to the screening invitation (non-responders)\(^3\). Non-participating women are at increased risk of cervical cancer, as half of the cervical carcinomas are found in this group of women\(^3\). Offering self-sampling methods has shown to improve attendance among the non-responders\(^38\). Detection of hrHPV in self-obtained cervico-vaginal samples is feasible, while cytological assessment of the self-sampler material is not reliable\(^39,40\).

**DNA methylation**

Abnormal patterns of DNA methylation have been recognized as frequent molecular changes in neoplasia\(^41\). DNA methylation occurs at the 5th position of a cytosine and only cytosines that are preceded by a guanine can become methylated. Promoter hypermethylation can result in transcriptional silencing of the gene. Methylation of tumor suppressor genes contributes to an immortalized phenotype by silencing expression of genes responsible for control of normal cell differentiation and/or inhibition of cell growth and has been reported to be an early event in carcinogenesis\(^41,42\). In addition to the functional implications of gene inactivation in tumor development, these methylation patterns represent excellent targets for diagnostic approaches\(^41\). Using bisulfite treatment, unmethylated cytosines are converted into uracil, but methylated cytosines are protected and remain cytosines. By taking advantage of the sequence differences, specific PCR primers can be designed that can distinguish the methylated DNA from unmethylated DNA by means of methylated specific PCR (MSP).

Quantitative methylation specific PCR (QMSP) is a specific and sensitive method that allows accurate quantification of methylation levels and high throughput analysis, making it suitable as a screening tool for (pre)malignant cervical neoplasia\(^43-45\). Methylation markers can be used as a primary screening test for cervical cancer, but also as a triage test using the same DNA as used for primary HPV testing.

To discover new cervical cancer specific DNA methylation markers, we followed a previous project where with pharmacological unmasking of hypermethylated silenced genes and expression microarray 4 cervical cancer specific methylation markers (C13orf18, Jam3, Epb41L3 and TERT) could be identified. These markers showed specificities for normal cervixes between 89-100% with corresponding sensitivities for detecting cervical cancer between 73-90%. However, the sensitivity for detecting CIN2 or higher lesions was only between 37-50%. For implementation of methylation analysis in population-based cervical cancer screening, a higher proportion of CIN2/3 needs to be detected.
Staging, treatment and prognosis of cervical cancer
Cervical cancer can be divided in different stages according the FIGO criteria (Table 2). During a bimanual gynecological examination under general anesthesia tumor size, involvement of vagina and parametrium and operability are assessed. The main histological types of cervical cancer are squamous cell carcinoma (80%) and adenocarcinoma (15%). Treatment is based on the FIGO stage; stage IB1, IB2 or IIA can be treated by surgery, while in all other stages concomitant chemoradiation therapy is the first choice of treatment. The 5-years survival depends upon the stage and varies from around 90% in stage 1 to 10% in stage 4. Different prognostic factors as tumor size, histological subtype, depth of stromal invasion, parametrial invasion and pelvic lymph nodes metastasis also determine the outcome of the patients. Locoregional recurrent disease after treatment remains a problem. Patient-tailored treatment with targeted drugs might be interesting for future perspectives. In this respect it would be interesting to find molecular markers that can predict response to chemoradiation.

Table 2. FIGO staging system

<table>
<thead>
<tr>
<th>FIGO stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>Stage I</td>
<td>The carcinoma is strictly confined to the cervix</td>
</tr>
<tr>
<td>IA</td>
<td>Invasive carcinoma, which can be diagnosed only by microscopy</td>
</tr>
<tr>
<td>IA1</td>
<td>Measured stromal invasion of ≤3.0 mm in depth and extension of ≤7.0 mm.</td>
</tr>
<tr>
<td>IA2</td>
<td>Measured stromal invasion of &gt;3.0 mm and ≤5.0 mm with an extension of ≤7.0 mm.</td>
</tr>
<tr>
<td>IB</td>
<td>Clinically lesions limited to the cervix uteri or preclinical lesions greater than stage IA</td>
</tr>
<tr>
<td>IB1</td>
<td>Clinically lesions ≤4.0 cm</td>
</tr>
<tr>
<td>IB2</td>
<td>Clinically lesion &gt;4.0 cm</td>
</tr>
<tr>
<td>Stage II</td>
<td>The carcinoma extending beyond the cervix but not to the pelvic sidewall or the lower third of the vagina.</td>
</tr>
<tr>
<td>IIA</td>
<td>Involvement of upper two-third of vagina, no parametrial invasion.</td>
</tr>
<tr>
<td>IIB</td>
<td>With obvious parametrial invasion</td>
</tr>
<tr>
<td>Stage III</td>
<td>The carcinoma extends to the pelvic wall and/or involves lower third of the vagina and/or causes hydronephrosis or nonfunctioning kidney</td>
</tr>
<tr>
<td>IIIA</td>
<td>Tumor involves lower third of the vagina with no extension to the pelvic wall</td>
</tr>
<tr>
<td>IIIB</td>
<td>Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney</td>
</tr>
<tr>
<td>Stage IV</td>
<td>The carcinoma has extended beyond the true pelvis or has involved the mucosa of the bladder or rectum</td>
</tr>
<tr>
<td>IVA</td>
<td>Spread of the growth to adjacent organs</td>
</tr>
<tr>
<td>IVB</td>
<td>Spread to distant organs</td>
</tr>
</tbody>
</table>
Outline of this thesis

To improve the current cervical cancer screening program, new biomarkers are necessary. Detection of different methylation patterns in the normal cervix and (pre)malignant cervical neoplasia might represent excellent diagnostic targets in new screening tests for detection of (pre)malignant cervical cancer. Many studies have been performed to find the ideal methylation marker that can identify (pre)malignant cervical neoplasia. In chapter 2 a systematic review is performed to summarize the results of studies analyzing methylation markers in cervical scrapings by (Q)MSP. An overview is given of the markers known in literature and the best methylation markers for cervical cancer screening reported so far.

Since the cervical cancer screening program in the Netherlands is going to change to primary hrHPV screening, the performance of the hrHPV test is of great interest. In chapter 3, the diagnostic performance of the widely-used Cervista HPV HR test is analyzed and compared to the Hybrid Capture 2 (HC2) test according to the International guidelines for HPV test requirements. In chapter 4 we show that the specificity of the Cervista HPV HR test can be further improved by changing the cut-off.

As we indicated in our systematic review a wide variety of methylation markers has been explored for cervical cancer screening, but so far no methylation markers are validated for optimal detection of (pre)malignant cervical neoplasia in a population based screening program. In chapter 5 we report an innovative genome-wide methylation analysis to identify new methylation markers that can differentiate between normal cervices and CIN2 or higher lesions.

Detection of hrHPV in self-obtained cervico-vaginal samples is feasible, while cytological assessment of self-sampler material is not reliable. Due to the relatively low specificity of the hrHPV test, an independent triage test is necessary. In chapter 6, the performance of DNA methylation analysis as triage test is compared with cytology in hrHPV positive women. For this purpose, we used the 4-gene panel C13ORF18, JAM3, EPB41L3 and TERT in a cohort of non-responders of the Dutch screening program. Furthermore, the feasibility of direct triage testing with DNA methylation analysis on brush-based self-sampled specimens is explored and compared to the DNA methylation results in the matched physician-taken samples.

(Chemo)radiation is standard of care for advanced stage cervical cancer patients. Unfortunately however, locoregional recurrence remains a frequent cause of death. To decrease locoregional recurrences adjuvant postradiation hysterectomy in patients with residual disease has been promoted, but its use is still extensively debated. In chapter 7 a retrospec-
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tive study is described in which the efficiency of post (chemo)radiation cervical biopsies to identify residual disease is evaluated. In patients with positive biopsies the possible impact of more radical surgery on locoregional recurrence frequency and treatment-associated morbidity is described as well.

Advanced stage cervical cancer patients that show marginal response to chemoradiation have poor prognosis. In response to DNA damage, caused by chemoradiation, cells can activate multiple stress- and damage-response pathways, including autophagy. Autophagy isolates and subsequently delivers cytoplasmic constituents for lysosomal degradation and is crucial in maintaining cellular integrity. Autophagy is initiated by the ULK1/ATG13 complex, and ATG13 is an important key player in this process. In chapter 8 we describe the role of ATG13-mediated autophagy in cervical cancer in response to radiation therapy. The summary of the results of the previous chapters are summarized in chapter 9 and chapter 10. Furthermore, in these chapters, future perspectives for cervical cancer screening are given.
References

2. The Dutch Cancer Registration. www.cijfersoverkanker.nl.


