New insights in methodology of screening for cervical cancer
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Chapter 1

General introduction
I. Cervical cancer

1.1. Epidemiology

Cervical cancer is the third most common cancer among women and the fourth leading cause of female cancer deaths. Each year, there are more than 529,000 new cases and around 275,000 deaths globally\(^1\). In the Netherlands, a low cervical cancer incidence and mortality rate country\(^2\), cervical cancer ranks as 11\(^{th}\) most frequent cancer among women, but the 3rd among women between 15 ~ 44 yrs. The latency data from World Health Organization/Institut Català d'Oncologia (WHO/ICO) Information Centre (2014) reported that ~750 women are diagnosed with cervical cancer and ~240 women die from the disease in the Netherlands each year\(^3\). In China, a populous and diverse country which covers more than 1.3 billion people and 9.6 million square kilometers, cervical cancer ranks as the 8\(^{th}\) most frequent cancer among women and the 2\(^{nd}\) in the age of 15~44 yrs. Data from WHO/ICO Information Centre (2014) showed, that each year ~61,500 new cases are reported and ~29,500 patients die of cervical cancer in China\(^4\). An explanation why China is a cervical cancer high incidence country, is the fact that a nationwide organized cervical cancer screening program is still lacking. The incidence and mortality of cervical cancer between different regions within China are variable (Table 1)\(^5\). Moreover, the existing cancer registries are geographically limited and the coverage of population is quite low, indicating that these data are probably not representative for the whole country.

1.2. Clinicopathology of cervical cancer

The most common cervical cancers are squamous cell carcinomas (SCC) and adenocarcinomas (ADC), which account for 75–90% and 10–25% of cervical cancers respectively\(^6,7,8\). A small proportion of cervical cancers (~3-5\%)\(^9\) represents adenosquamous carcinomas and other rare histological types including neuroendocrine carcinomas\(^10\).

The cervix uteri consists of the ectocervix and endocervix. The ectocervix is mainly lined with non-keratinizing stratified squamous epithelium and the endocervix with mucus producing columnar epithelium. The cells in the squamocolumnar junction (SCJ) termed as transformation zone are less stable and particularly susceptible to viral infections\(^11\). SCC most often occurs at the SCJ between the ecto- and endocervix.
Table 1. The database of the incidence and mortality of cervical cancer in China  
(data adapted from Shi et al$^5$)

<table>
<thead>
<tr>
<th></th>
<th>Database</th>
<th>Cover national population</th>
<th>Rate (Per 100,000)</th>
<th>Sites/Regions</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incidence</strong></td>
<td>International Agency for Research on Cancer's (IARC’s) 1993-2002</td>
<td>≤ 2% females</td>
<td>1.2-4.6</td>
<td>Shanghai, Jiashan, Tianjin, Beijing, Wuhan, Qidong, Harbin, Zhongshan and Guangzhou</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chinese National Center for Cancer Registries (NCCR) system 1998-2003</td>
<td>≤ 6%</td>
<td>1.9-8.4</td>
<td>30–37 registry sites</td>
<td>&gt;10 /10$^5$ Yangcheng in Shanxi province &amp; Shenzhen in Guangdong</td>
</tr>
<tr>
<td></td>
<td>NCCR 2004-2006</td>
<td>≤ 6%</td>
<td>6.0-9.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mortality</strong></td>
<td>national retrospective survey 1975</td>
<td>14.6</td>
<td>11 sites</td>
<td>(overestimated)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>national retrospective survey 1990–1992</td>
<td>~10%</td>
<td>4.3</td>
<td>11 sites</td>
<td>(underestimated)</td>
</tr>
<tr>
<td></td>
<td>national retrospective survey 2004–2005</td>
<td>~10%</td>
<td>2.5</td>
<td>11 sites</td>
<td>(underestimated)</td>
</tr>
<tr>
<td></td>
<td>Chinese National Center for Cancer Registries (NCCR) system 1998-2003</td>
<td>≤ 6%</td>
<td>1.2 ~ 7.0</td>
<td>30–37 sites</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center of Health Information and Statistics (CHIS) reporting system,WHO</td>
<td>~1%</td>
<td>2.7 (overall)</td>
<td>age-standardized</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDC $^71$</td>
<td>~10%</td>
<td>4.2 (rural)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.8 ( urban )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
and is therefore detected easier by cytologic screening. In contrast, ADC develops from the gland cells within the endocervical canal. Consequently, ADC is mainly diagnosed at an advanced stage. Compared to SCC, ADC associates with a worse prognosis, is less sensitive to radiation therapy and chemotherapy, and easily tends to metastasize. Therefore, overall patients with ADC have a 5-years survival rate that is 10-20% lower than SCC\textsuperscript{8,12}.

1.3. Treatment of cervical cancer

According to the International Federation of Gynecology and Obstetrics (FIGO) stage, microinvasive cervical cancer can be treated by large loop excision of the transformation zone (LLETZ) or cone biopsy. Early stage tumors can be managed by radical hysterectomy plus pelvic lymph node dissection or (chemo)radiotherapy. Advanced stage tumors are almost always treated by (chemo)radiotherapy. Five year survival approaches 100% for patients with tumors of stage IA and averages 70–85% for those with stage IB1 and small IIA lesions, 50–70% for stages IB2 and IIB, 30–50% for stage III and 5–15% for stage IV\textsuperscript{13}. Therefore, early detection is one of the most effective strategies for saving cervical cancer patient lives.

1.4. Cervical precursor lesions

1.4.1 Histologic and cytologic classification

Histologically, the premalignant lesion of SCC is referred to as cervical intraepithelial neoplasia (CIN). Based on the severity of the changes and especially on the proportion of the epithelial layer with neoplastic changes, CIN is stratified in 3 grades, CIN1, CIN2 and CIN3 representing 1/3, 2/3 and almost the total layer of epithelium containing atypical cells\textsuperscript{14}. Routine cytological classifications are the Pap and Bethesda systems (Table 2, Figure 1). According to the severity of abnormal smears, the European Pap classification is graded into 5 classes, PAP1 to PAP5\textsuperscript{15} (Table 2, Figure 1). The American Bethesda classification was developed to differentiate between lesions probable to progress to cervical cancer (high-grade squamous intraepithelial lesions (HSIL)) and lesions less probable to progress (low-grade squamous intraepithelial lesions (LSIL))\textsuperscript{16}. In Table 2 and Figure 1, the correspondence between the histologic and cytologic classifications is illustrated.
Table 2: Overview of the different nomenclatures and classifications for histologic and cytologic cervical abnormalities  
(These data were adapted from the thesis of Nan Yang: Detection of DNA Hypermethylation as a Diagnostic Tool in Cervical Neoplasia (ISBN978-90-367-4169-9))

<table>
<thead>
<tr>
<th>Histology</th>
<th>Cytology</th>
<th>Bethesda</th>
<th>Papanicolaou</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>Within normal limits</td>
<td>Pap 1</td>
</tr>
<tr>
<td>Benign atypia</td>
<td>Inflammatory atypia</td>
<td>Benign cellular changes</td>
<td></td>
</tr>
<tr>
<td>Atypical cells</td>
<td>Squamous atypia</td>
<td>ASCUS</td>
<td>Pap 2</td>
</tr>
<tr>
<td>Mild Dysplasia</td>
<td>CIN1</td>
<td>Low-grade SIL (LSIL)</td>
<td>Pap 3A1</td>
</tr>
<tr>
<td>Moderate Dysplasia</td>
<td>CIN 2</td>
<td>High-grade SIL (HSIL)</td>
<td>Pap 3A2</td>
</tr>
<tr>
<td>Severe Dysplasia</td>
<td>CIN 3</td>
<td></td>
<td>Pap 3B</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td></td>
<td></td>
<td>Pap 4</td>
</tr>
<tr>
<td>(Microinvasive) cancer</td>
<td>(Microinvasive) cancer</td>
<td>(Microinvasive) cancer</td>
<td>Pap 5</td>
</tr>
</tbody>
</table>

![Figure 1: The development of cervical carcinogenesis (Adapted from Snijders et al 2006).](image)

The precursor of invasive ADC was first described by Friedell and McKay\textsuperscript{17} in 1953 as adenocarcinoma in situ (AdCIS). Histologic features of AdCIS include preservation of normal glandular architecture and partial or complete involvement of endocervical
glands and abrupt transition to normal endocervical epithelium. The cytoplasm can be depleted or abundant, vacuolated, granular, and basophilic or eosinophilic\textsuperscript{18,19}. The 3 most frequent AdCIS subtypes are endocervical (usual), intestinal, and endometrioid\textsuperscript{19}. Unlike CIN, AdCIS is much less frequent and also more difficult to detect effectively. AdCIS is not well visualized colposcopically as it can arise high up in the endocervical canal\textsuperscript{20} and the cytomorphologic criteria for identifying neoplastic glandular lesions are not as well defined as for CIN. Additionally, AdCIS frequently coexists with squamous intraepithelial lesions or squamous cell carcinoma in 50% of cases\textsuperscript{18}. Therefore, the detection of premalignant ADC lesions in scrapings is more difficult compared to premalignant SCC lesions.

1.4.2 Treatment of precursor lesions

Most cases of CIN1 will regress spontaneously, while up to 50% of the CIN3 lesions may progress to cervical cancer, when left untreated. Both in the Netherlands as well as in China, all identified CIN2 and CIN3 lesions are treated, because it is still not possible to identify those lesions that are most likely to progress. Treatment options for CIN2 and CIN3 are LLETZ, cryocoagulation, laser evaporation, cone biopsy or in rare cases hysterectomy. In general, LLETZ is the preferred treatment for CIN2 and CIN3, because it is simple, cheap, effective and allows pathologic examination of the specimens\textsuperscript{21}.

II. Cervical cancer screening program

Cervical cancer development takes a long time in most patients. Normally, HSIL (CIN2 and CIN3) develop within 3–5 years following a high-risk HPV (hrHPV) infection, whereas further progression to invasive cancer can take up to 20–30 years\textsuperscript{10}. This long period offers many opportunities for intervention and prevention of cervical cancer. Based on previous large-scale studies, a systematic routine screening program can reduce the incidence of cervical cancer by at least 60% and has been recommended by WHO, particularly in developing countries\textsuperscript{22}. Recently, a meta-review collected all the available evidence in literature, which also supports the notion that cervical screening does offer protective benefits and is associated with a reduction in the incidence of invasive cervical cancer and cervical cancer mortality\textsuperscript{23}.
In the Netherlands, pilot-studies on population-based screening by cytomorphologic classification were started in the 1970s. A nationwide screening program aimed at specific age categories was initiated in 1988. Between 1988 and 1996, women aged 34–54 years were screened once every 3 years; and from 1996 onwards, women aged 30–60 were screened every 5 years\(^2^4\). The current Dutch screening program is primarily based on cytologic examination of cervical smears with hrHPV testing as a triage test for abnormal cytologic results (ASCUS/LSIL)\(^2^5\). In the Netherlands, the population-based screening program will change to primary hrHPV screening in 2016\(^2^6\).

In China, cervical cancer screening remains opportunistic screening. Some women have access to population-based or employee-based screening through large corporations or organizations. A first pilot screening program named the Cervical Cancer Screening Study (SPOCCS) project was performed in Shanxi Province, a region with a heavy burden of cervical cancer\(^2^7,2^8\). After that, followed by the “Program of Cancer Prevention and Control in China (2004-2010)”, the government took the “Top Down” planning approach, i.e. set-up of two demonstration sites as potential models for cervical cancer control. One was in a poverty-stricken county in Shangxi\(^2^9\) with a high cervical cancer incidence and applies simple, inexpensive technologies to screen for precancerous lesions. Another one was in Shenzhen\(^3^0\), a prosperous city with high-resource settings. With the experience from the two regions, cervical cancer screening sites were increasingly expanded to a total of 31 cities, 43 sites, autonomous regions included as well\(^3^1\). One of the challenges for China is that 70% of the population is rural and 85% of the cervical cancers occur in the less developed regions. Therefore, a government-sponsored program proposed by the All-China Women's Federation for free cervical cancer screening in women age 35-59 years has been setup in July 2009\(^3^2\). During 2009-2011, 10 million rural women were screened by either Pap smear or visual inspection with acetic acid (VIA). However, in light of an estimated 500 million women in China, the coverage is still too low. Therefore, in 2012, the program was extended over the next three years and was offered to more rural women\(^3^3\). Among other hurdles, China lacks a sufficient number of cytopathologists or trained health-care workers to screen all of these women by Pap smear or VIA, respectively. A nationwide screening round needs to...
be followed by development of more appropriate health-care policies and services for all Chinese women.

Currently, The Ministry of Health of China has launched the Guideline for Screening and Early Detection and Treatment of Cervical Cancer\textsuperscript{34,35}. According to the guideline, the target population is women > 21 years old or with sexual intercourse experience for more than three years. Depending on the diverse geographical socioeconomic status and levels of exposure to the risks of the population, the guideline has recommended three protocols with a different combination of methodologies: i) primary screening by liquid-based cytology test plus HPV DNA test in developed regions and/or women with good economic status; ii) primary screening by Pap smear cytology test plus HPV DNA test in moderately developed regions; iii) primary screening by VIA in low-resource settings\textsuperscript{35}.

\textbf{III. Cervical cancer screening approach}

\textit{3.1 Visual inspection with acetic acid (VIA)}

VIA, a “screen-and-treat” method, has been suggested to be performed in the less-developed countries and regions. The principle of VIA is that most CIN2 and CIN3 lesions are acetowhite (i.e. they develop a white color when vinegar or 5% acetic acid is applied to the cervical epithelium that harbors preinvasive lesions), while the normal cervical epithelium maintains its pink color after application of 5% acetic acid\textsuperscript{36}. VIA is a point-of-care test, producing immediately results, also allowing consecutive treatment eg. by cryocoagulation. Most importantly, VIA does not require a cytopathology laboratory and therefore can be performed with little infrastructure and low costs. Although VIA sounds quite simple, it is actually more complicated than it appears. Acetowhitening is a relatively non-specific cervical finding. Many women have acetowhitening of the cervix not because of CIN2 and CIN3 lesions but immature squamous metaplasia or reparative conditions etc. In case of insufficient quality control, VIA will lead to over-treatment of many women who do not have CIN2+ lesions\textsuperscript{36}.
3.2 Morphology-based testing
Generally, all the morphology-based testing methods such as automated Pap-smear analysis or liquid-based cytology show the same limitations, i.e. less sensitivity (50~60%) and subjective interpretations. In addition, these techniques require training, the development of laboratory infrastructure, standardization and quality control measures. Furthermore, there is a disputable problem that a relevant amount of high-risk cases from patients living in low resource regions may be missed due to low sensitivity techniques of the screening protocols. Therefore, there is ample room for improvement.

3.3 HPV as a marker for cervical cancer screening
The strong association of hrHPV infection with cervical cancer is widely known. HPV is a genus of the family Papovaviridae. The HPV virions are non-enveloped and icosahedral with a circular double stranded DNA genome of almost 8kb in length. Its genome can be divided into three different regions: (i) a coding region containing the seven early genes E1~ E7; (ii) a region containing the late genes encoding capsid proteins; and (iii) a non-coding region, termed the long control region (LCR). Expression of the viral proteins E6 and E7 is crucial for cervical carcinogenesis, E6 binds to p53 and herewith inactivates p53-mediated growth suppression and apoptosis, whereas E7 binds to Rb which inactivates this negative regulator of the cell cycle. It is well established that a series of subsequent steps after HPV infection results in progression to cancer: HPV persistence, HPV-mediated epithelial transformation, development of precancerous lesions (CIN1-3) and then invasive cervical cancer.

So far, more than 150 HPV types have been isolated and sequenced. Table 3 illustrates the main genera and their association with human diseases. According to their oncogenic potential, the mucosal (alpha) HPV types are divided into two groups: low-risk HPV (lrHPV), which are mainly associated with benign genital warts, and high-risk HPV (hrHPV), which are the etiological agents of cervical cancer. So far within the discovered cancer related HPVs, 12 types of HPV (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58 and HPV59) have now been consistently classified as hrHPV (also known as International
Table 3. The main diseases associated with different HPV types

(Adapted from Tommasino et al 201339).

<table>
<thead>
<tr>
<th>HPV family</th>
<th>Pathogenicity</th>
<th>HPV type</th>
<th>Disease (% attributed cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Mucosal high-risk</td>
<td>HPV16</td>
<td>Cervical squamous cell carcinoma (~50%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cervical adenocarcinoma (~35%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oropharyngeal cancer (~25%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV18</td>
<td>Cervical squamous cell carcinoma (~20%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cervical adenocarcinoma (~35%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV31,33,35,39,45,51,52,56,58,59</td>
<td>Cervical squamous cell carcinoma (~30%)</td>
</tr>
<tr>
<td></td>
<td>Mucosal low-risk</td>
<td>HPV6,11</td>
<td>Benign genital lesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Respiratory papillomatosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV13,32</td>
<td>Oral focal epithelial hyperplasia</td>
</tr>
<tr>
<td>μ</td>
<td>Cutaneous</td>
<td>HPV2,3,27,57</td>
<td>Skin warts</td>
</tr>
<tr>
<td></td>
<td>benign</td>
<td>HPV1</td>
<td>Skin warts</td>
</tr>
<tr>
<td>β</td>
<td>Cutaneous</td>
<td>HPV5 and 8</td>
<td>First β HPV types isolated from SCC of EV individuals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV9,12,14,15,17,19-25,36-38,</td>
<td>Likely associated with SCC in EV patients as well as</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47,49,75,76,80,92,93,96,98-100,104,105,107,110,111,113,</td>
<td>immuno-compromised and immune-competent individuals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>115,118,120,122,124,143,145,150-152,159</td>
<td></td>
</tr>
</tbody>
</table>
Agency for Research on Cancer (IARC) class I), HPV68 has been classified as probable high-risk (also known as IARC class 2A), and another seven types have been classified as possible high-risk (HPV26, HPV53, HPV-66, HPV67, HPV70, HPV73 and HPV82, also known as IARC class 2B)⁴⁰.

Globally, HPV16 and -18 are the most oncogenic genotypes, which are related to about 70% of invasive cervical carcinoma, 50% of high-grade lesions (CIN2/3) and 35% of CIN1. However, the prevalence and distribution of HPV genotypes show considerable geographic and ethnic variation, especially for the less common types. **In the Netherlands**, about 4% of women in the general population are estimated to harbor cervical HPV infection in their life time, and 82.3%, 70.2% and 22.7% of respectively invasive cervical cancers, HSIL and LSIL are attributed to HPV16 or HPV18. The most common HPV types in CIN are HPV16, HPV31 and HPV18³. **In China**, in the general population about 13.7% of women⁴ (ranging from 6.7% to 45.6% in different studies (Table 4)), are estimated to harbor HPV infection in cervical scrapings, of which 75.5%, 44.2% and 23.1% of invasive cervical cancers, HSIL and LSIL, respectively, are attributed to HPV16 or HPV18. In contrast to the Netherlands, in China the most common types in CIN are HPV16, HPV52, HPV58⁴. The HPV prevalence in women from population-based screening studies in China in different time periods are listed in Table 4. Although so far there are several programs including HPV testing for screening of cervical cancer and their precancerous lesions, at present these programs cover only a small part of China presently representing ~9 provinces/cities and a population of ~134,000 women (Figure 2). HPV testing is playing an increasing role in cervical screening.

Currently, 7 tests have been approved by the United States Food and Drug Administration (FDA). The first reliable, quality standardized HPV DNA test is the Hybrid Capture (HC) assay, as developed by Digene Corporation (Gaithersburg, MD, USA), which gained FDA approval in 1999. In 2003, FDA approval was extended to the use of Hybrid Capture 2 (HC2) in Pap testing. Later, the HC2 test, which detects 13 hrHPV was recognized as “golden standard” and widely applied today. However, some limitations, for instance, cross-reactivity and lack of control for input DNA,
Table 4. HPV prevalence in women from population-based screening studies in different time period

(Adapted from Li et al, 2013[72])

<table>
<thead>
<tr>
<th>Author / Pub year</th>
<th>Study year</th>
<th>Location (County/city, Province)</th>
<th>Number (N)</th>
<th>Age Range (yrs)</th>
<th>Lab-assays</th>
<th>overall HPV prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belinson et al., 2001[73]</td>
<td>1999</td>
<td>Xiangyuan</td>
<td>1,997</td>
<td>35-45</td>
<td>HC2</td>
<td>18.2</td>
</tr>
<tr>
<td>Shen et al., 2003[74]</td>
<td>2001-2002</td>
<td>Xiangyuan &amp; Yangcheng, Shanxi</td>
<td>9,683</td>
<td>30-50</td>
<td>HC2</td>
<td>27.5</td>
</tr>
<tr>
<td>Zhao et al., 2006[75]</td>
<td>2001-2002</td>
<td>Xiangyuan &amp; Yangcheng, Shanxi</td>
<td>8,798</td>
<td>35-50</td>
<td>HC2</td>
<td>23.6</td>
</tr>
<tr>
<td>Zou et al., 2011[76]</td>
<td>2004</td>
<td>Yangcheng</td>
<td>745</td>
<td>15-59</td>
<td>HC2</td>
<td>16.0</td>
</tr>
<tr>
<td>Li et al., 2007[77]</td>
<td>2004</td>
<td>Xiusi, Jiangxi</td>
<td>2,432</td>
<td>30-49</td>
<td>HC2</td>
<td>18.5</td>
</tr>
<tr>
<td>Li et al., 2006[78]</td>
<td>2004-2005</td>
<td>Shenyang, Liaoning</td>
<td>685</td>
<td>15-59</td>
<td>GP5+/6+ mediated PCR</td>
<td>16.8</td>
</tr>
<tr>
<td>Wu et al., 2007[80]</td>
<td>2004-2005</td>
<td>Shenzhen, Guangdong</td>
<td>1,027</td>
<td>15-59</td>
<td>GP5+/6+ mediated PCR</td>
<td>16.6*</td>
</tr>
<tr>
<td>Wu et al., 2013[81]</td>
<td>2006-2007</td>
<td>Shanxi, Beijing, Xinjiang, Henan, Shanghai</td>
<td>4215</td>
<td>17-54</td>
<td>HC2</td>
<td>14.3</td>
</tr>
<tr>
<td>Li et al., 2008[82]</td>
<td>2006</td>
<td>Chaozhou, Guangdong</td>
<td>1705</td>
<td>20-68</td>
<td>PCR (MY09/11 primer)</td>
<td>9.03</td>
</tr>
<tr>
<td>Zhao et al., 2009[83]</td>
<td>2006-2008</td>
<td>Beijing</td>
<td>5,552</td>
<td>25-54</td>
<td>PCR (MY09/11 primer)</td>
<td>6.7</td>
</tr>
<tr>
<td>Li et al., 2010[84]</td>
<td>2006-2009</td>
<td>Beijing</td>
<td>6,185</td>
<td>25-54</td>
<td>HC2</td>
<td>9.9</td>
</tr>
<tr>
<td>Ye et al., 2010[85]</td>
<td>2007-2008</td>
<td>Zhejiang</td>
<td>5,058</td>
<td>20-79</td>
<td>PCR (MY09/11 primer)</td>
<td>13.3</td>
</tr>
<tr>
<td>Wang et al., 2012[86]</td>
<td>2007-2010</td>
<td>Liaoning</td>
<td>24,041</td>
<td>18-60</td>
<td>PCR (MY09/11 primer)</td>
<td>45.6</td>
</tr>
<tr>
<td>Wu et al., 2010[87]</td>
<td>2008-2009</td>
<td>Fujian</td>
<td>2,338</td>
<td>20-70</td>
<td>PCR (MY09/11 primer)</td>
<td>22.5</td>
</tr>
<tr>
<td>Hu et al., 2011[88]</td>
<td>2009</td>
<td>Jiangsu</td>
<td>316</td>
<td>18-25</td>
<td>HC2</td>
<td>17.1</td>
</tr>
<tr>
<td>Chen et al., 2012[89]</td>
<td>2009-2010</td>
<td>Chaozhou, Guangdong</td>
<td>48,559</td>
<td>35-60</td>
<td>Multiplex realtime RCR</td>
<td>7.89</td>
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<tr>
<td>Zhang et al., 2013[90]</td>
<td>2011</td>
<td>Shanghai suburb</td>
<td>10,000</td>
<td>17-89</td>
<td>PCR (MY09/11 primer)</td>
<td>12.6</td>
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*HPV overall prevalence including high-risk HPV and low-risk HPV
results in false negative and positive results. The second HPV testing platform, Cervista HPV HR test (Hologic, WI, USA) detects the same 13 hrHPV subtypes as HC2 and includes HPV66 as well. The Cervista HPV16/18 test was the first FDA approved assay which permits additional genotyping. In 2014, the Cobas 4800 HPV test (Roche Molecular Diagnostics, Pleasanton, CA, USA) detecting not only the same 13 hrHPV but also discriminating between HPV16, HPV18 and the other hrHPV, was FDA-approved for primary HPV screening. Finally, the APTIMA HPV assay (Hologic formerly GenProbe Inc., San Diego, CA, USA) for the detection of E6 and E7 hrHPV mRNA was approved by FDA. In addition to the FDA approved HPV detection tests, more than 50 other in-house and commercial HPV-tests are available. Therefore, it is a real challenge to choose the most reliable HPV assay for primary cervical HPV screening. In 2009, in an international guideline the criteria were reported for a candidate hrHPV test to be used for primary HPV screening. A new test should be ‘non-inferior’ with respect to clinical sensitivity (≥90%) and
specificity (≥98%) for the detection of CIN2+ when compared with the clinically validated HC2 assay in women aged ≥30 yrs. The guideline also describes the requirements of the technical robustness of new assays through the measurement of intra- and inter-laboratory reproducibility.

Because of the HPV natural history, most of HPV infections are usually temporary, in around 91% of women with an HPV infection, HPV is already cleared within two years. However, despite the clearance of HPV infections in most women, especially in the younger, sexual active population, HPV is present in virtually all of these women at a certain time. Since HPV testing does not discriminate between temporary and persistent HPV infections, the specificity of the test to detect CIN2+ lesions is low. Therefore, despite of the superior sensitivity of HPV testing, the lower specificity is an inevitable problem leading to a need to discover novel biomarkers for cervical cancer screening for triage testing. P16\textsuperscript{INK4a} and Ki67 are established cell cycling biomarkers and described as a direct marker of HPV infection\textsuperscript{43,44}. A recent study reported that a combined dual-stained cytology test for both p16\textsuperscript{INK4a} and Ki67 had a sensitivity of 91.9% for detecting CIN2+ and 96.4% for CIN3+. This test was also highly specific: 82.1% for CIN2+ and 76.9% for CIN3+\textsuperscript{45,46}. Another biomarker, ProExC, is a cocktail of MCM2 and TOP2A proteins\textsuperscript{47}, which might be a sensitive and specific marker for distinguishing CIN2/3 from metaplastic squamous epithelium\textsuperscript{47}. However, there is insufficient evidence to integrate these strategies into the standard of care for cervical cancer screening and large screening trials are still needed to validate these biomarkers\textsuperscript{48}. More importantly, these immunohistochemistry-based triage tests cannot be performed on the DNA extracted for HPV testing and thus need different handling in the lab, are microscopy-dependent, require a well-fixed specimen with preserved morphology and skilled cytologic/histologic pathologists\textsuperscript{10}.

3.4. DNA methylation as a biomarker for cervical cancer screening

In 1942, C. H. Waddington named a word “epigenetic” when he was studying the causal interactions between the genotype and the phenotype\textsuperscript{49}. Now epigenetics refers to heritable modifications of the genome without any changes in primary DNA sequences including DNA methylation, histone modifications, chromatin remodeling, and noncoding RNAs\textsuperscript{50}. Among them, DNA methylation is the best characterized so
far. The modification is a covalent modification of DNA, in which a methyl group is transferred from S-adenosylmethionine (SAM) to the fifth position of the pyrimidine ring of cytosine catalyzed by DNA methyltransferases (DNMTs)\(^5^1\) \(^5^2\) (Figure 3A). DNA methylation occurs only at cytosines located 5’ to guanosine in the CpG dinucleotide\(^5^3\) (Figure 3B). Genomic CpG islands are nucleotide regions where the percentage of the CpG dinucleotides is higher. Hypermethylation of CpG islands within the promoter region is correlated with transcriptional silencing\(^5^4\) \(^5^5\) (Figure 3C).

Abnormal DNA methylation is a well-recognized epigenetic hallmark of cancer cells and has been observed in most malignancies. The first aberrant methylation alterations in human cancer was discovered by Feinberg and Vogelstein in 1983\(^5^6\). Cancer often exhibits hypermethylation at the promoter region with simultaneous widespread hypomethylation from normal methylated sites (Figure 3C). Promoter DNA methylation is an early event in cancer development\(^5^7\). Furthermore, its patterns are regulated in developmental stage specific, cell type specific and tissue-specific manner supporting the idea that DNA methylation analysis might be a valuable biomarker for cancer screening and early detection.

Although infection with hrHPV is a necessary feature, HPV in itself is not sufficient for development of cervical cancer. It is known that integration of the viral DNA into the cellular genome causes not only genetic but also epigenetic alterations. These result in the silencing of tumor suppressor genes (TSG) and the overexpression of oncogenes\(^5^8\). As for HPV\(^5^9\), also aberrant methylation patterns of cancer-associated genes have been observed throughout the process of cervical carcinogenesis\(^5^9\), \(^6^0\),\(^6^1\). Therefore, in order to satisfy the requirements from clinical practice, the research on DNA methylation biomarkers for molecular diagnostics encourages the translation of this field from the bench to clinical practice.

By 2009, more than 68 genes had been analyzed for methylation in cervical tissues and/or scrapings representing various stages of cervical cancer development as reviewed by Wentzensen et al\(^6^2\). From this compilation of markers, the authors concluded that three markers (DAPK, CADM1 and RARB2) consistently showed elevated methylation in cervical cancers. The low concordance between studies for
Figure 3. A) Cytosine methylation: Methylation of the 5th position on cytosine reveals the most common methylated residual described as 5-methyl-cytosine or 5-mC. Catalyzed by DNMTs, a methyl-group is at the 5th position of cytosine in the presence of SAM, then SAM is converted to SAH. (Adapted from http://www.intechopen.com/books/methylation-from-dna-mrna-and-histones-to-diseases-and-treatment/dna-methylation-stem-cells-and-cancer)

B) DNA methylation occurs only at cytosines located 5’ to guanosine in the CpG dinucleotide sequences. (Adapted from http://jonlieffmd.com/blog/networks-of-genes-respond-to-social-experiences)

C) Methylation of CpG islands within the promoter region is associated with gene inactivation. (Adapted from http://missinglink.ucsf.edu/lm/genes_and_genomes/methylation.html)

the other genes most likely reflects the use of different assays, assay thresholds and/or selected promoter regions that were analyzed. However, methylation results obtained from tissue samples may not be directly extrapolated to cervical scrapings. The difference in cell type composition may display distinct levels of background methylation. Apart from samples error, some other limitations still hold back the process for the DNA methylation markers applied in the clinical practice. First of all, by application of single methylation markers on cervical scrapings, sensitivities for cervical cancer of 90% or more has been achieved; however, the positivity rate for HSIL and CIN2 is generally lower. Secondly, the combined methylation analysis of more genes in a panel has resulted in markedly increased sensitivity for HSIL. Using cervical scrapings of referral populations, sensitivities of over 80% for CIN3+ were obtained with marker panels, JAM3/EPB41L3/TERT/C13ORF18.
SOX1/PAX1/LMX1A/NKX6-1\textsuperscript{66} and CADM1/MAL\textsuperscript{67}, but these findings need further validation\textsuperscript{10,63}. Third, because of the difficulty in early detection of cervical AdCIS by morphologic-based method, methylation of certain genes might be more specific for cervical AdCIS. So far, few studies have focused on the DNA methylation associated with ADC. Recently one study reported methylation of PAX1, PTPRR, SOX1 and ZNF582 in ADC\textsuperscript{68}. However, validation in a large set of samples is lacking.

These years, advances in whole genome profiling technologies have revolutionized the field of cancer research. In our previous studies\textsuperscript{69}, our approach to identify new methylation markers were based on a pharmacological unmasking expression microarray approach to enrich for genes that are silenced and re-expressed during functional reversal of DNA methylation upon treatment with demethylation agents. Nevertheless, antibody-based MeDIP is always variable and microarray-based screening has the drawbacks including their design, production and somehow the inaccurate hybridization signals. The use of next-generation-sequencing platforms to identify novel methylation markers with more sensitivity and accuracy compared to traditional microarray profiling seems very promising\textsuperscript{70}. These technologies have facilitated the discovery of potential biomarkers for cervical cancer development and progression as demonstrated in this thesis.

**Outline of this thesis**

Currently, the HPV-based cervical cancer screening program is going to be as primary testing in order to substitute the lower sensitivity and subjective interpretation of cytomorphology-based screening in several European countries including the Netherlands. However, the method applied for HPV DNA detection should fulfill the criteria that are recommended in the international guideline. In addition, although high sensitivity is obviously advantage of HPV-based screening, the lower specificity is not, because it will result in relatively more referrals for colposcopy despite the lack of CIN2+ lesions. Therefore, there is still room to improve the early diagnosis of (pre)malignant disease in cervical scrapings, especially in the discovery of biomarkers with both high sensitivity and specificity. Compared to microarray-based
methods with defined potential methylation regions, the use of MethylCap-Seq, a new innovative method based on a genome-wide screening approach resulting in a specific methylome of the analyzed sample, could be promising to identify novel biomarkers.

In China studies on the prevention of HPV-related disease, especially cervical cancer is becoming more important over the last few years. At this moment, most of the reports regarding the prevalence of HPV infection and the distribution of genotypes in China were generated with scrapings obtained from either hospital-based populations or collected in few provinces/cities. The precise information on the occurrence of cervical HPV infection on the national level is still absent. In order to lay the basis for the future HPV-based screening and vaccination, in Chapter 2 the epidemiological data regarding the correlation of the HPV prevalence and genotype with the age and regional distribution in China were analyzed on total 120,772 clinical cervical samples collected in 37 cities of China in 2012.

The Cervista™ HR HPV test was the second hrHPV assay approved by the FDA. In comparative studies, Cervista showed similar sensitivity and specificity as the HC2 test. However, Cervista has not previously been formally validated for the use in primary cervical screening according the international guideline, especially in Chinese population. Hereby, in Chapter 3, the clinical sensitivity and specificity of the Cervista™ HR HPV test were compared to that of HC2 for detection of high-grade cervical lesions (CIN2+) in women aged ≥30 years in >7,000 screening samples selected from a Chinese population-based SHENCCAST II dataset by non-inferiority analysis following the international guideline. In addition, the intra- and inter-laboratory reproducibility of Cervista in 510 scraping following the international guideline was determined.

In clinical practice, early diagnosis in the precancerous stage is most important for cervical cancer prevention. Therefore, the aim of population-based screening for cervical cancer is to detect all CIN 2/3 lesions. However, so far, no methylation markers, including those identified by our own group previously, have been reported to be sufficient sensitive and specific for the detection of CIN 2/3 lesions. In Chapter
4, a new approach to discover new methylation markers specific for high-grade cervical intraepithelial neoplasia (CIN2/3) was used based on innovative genome-wide methylation analysis (MethylCap-Seq) by comparison of the methylome of 20 normal cervices with 18 CIN2/3 lesions, followed by the validation of the diagnostic performance of several new candidate methylation markers in cervical scrapings.

Currently, the incidence of SCC is declining in most developed countries. In contrast, there is a rise in the absolute and relative incidence of cervical ADC, especially in younger women. One explanation for the increase of ADC is the less effective cytomorphologic-based detection of ADC in population-based screening programs. DNA methylation markers might be of help to improve the diagnostic limitations. However, no specific methylation markers have been described for the detection of ADC. In Chapter 5, MethylCap-Seq analysis was performed by comparison of the methylome of 20 normal cervices with 6 ADC and 6 SCC to identify methylation markers with a high sensitivity for both ADC and SCC, followed by the validation of the diagnostic performance of several new candidate methylation markers in cervical scrapings.

The results of chapters 2 to 5 will be summarized in Chapter 6 including some future perspectives.

Reference


Chapter 1


9 Cervical cancer: Epidemiology, aetiology, pathogenesis and main histological types. .


General introduction

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