Detection of DNA hypermethylation as a diagnostic tool in cervical neoplasia

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CHAPTER 7

Summary and future perspectives
Summary
Cervical cancer is the second most common malignancy among women worldwide (1). In the Western world, cervical cancer accounts for only 3.6% of new cancers in women. In developing countries however cervical cancer accounts for 15% of newly diagnosed cancers and is a major cause of death (2). Cervical cancer is an important cause of early loss of life as it affects relatively young women. Five year overall survival approaches 100% for patients with stage IA tumors and averages 70–85% for those with stage IB1 and smaller IIA lesions. However, five year disease-free survival is only 50–70% for stages IB2 and IIB, 30–50% for stage III, and 5–15% for stage IV (3). Early detection of cervical cancer by population-based cytomorphological screening of cervical scrapings has been introduced in most developing countries and resulted in a significant decrease in the cervical cancer incidence by detection of its precursors. Cervical cancer originates from its precursors, cervical intraepithelial neoplasia (CIN). CIN I regresses in most cases, while 25-50% of CIN II and III could progress to cervical cancer when left untreated (4). It is estimated that the progression from CIN to cervical cancer generally takes a decade (5). The mean age of women with CIN lesions is 35 years, with a peak incidence of 3550 per 100,000 women at age 29 (6). Cervical cytological changes can be detected in an early and pre-invasive stage of the disease. Detection and treatment of these precursor lesions can effectively prevent cervical cancer (7).

Current cervical cancer screening is based on cytomorphological assessment of cervical smears. Due to sampling and screening errors, the number of false negative and false positive screening results is substantial, leading to an overshoot of diagnosis of cervical cancer (8-10). New methods based on molecular changes that occur during cervical carcinogenesis, should theoretically improve the current approach. One of these new methods is DNA methylation analysis. An overview of different techniques to detect DNA methylation is given in chapter 2. The advantages and disadvantages of all these techniques are discussed. Methylation specific PCR (MSP) still is the most popular technique to detect methylation. Compared to conventional MSP, real-time quantitative MSP (QMSP) is more sensitive and specific. Furthermore, it is a high-throughput technique, which could make QMSP suitable for implementation in nation-wide screening programs.

In previous studies, many gene promoters have been identified as being aberrantly methylated in cervical cancer, however most of these studies did not include precursor lesions in their analysis (11-19). In chapter 3 more insight is obtained in the course of methylation throughout cervical carcinogenesis by performing QMSP on biopsies from 20 normal cervices, 20 CIN I, 20 CIN II/III and 60 cervical cancers and on corresponding scrapings from 9 normal cervices, 11 CIN I, 18 CIN II/III and 20 cervical cancers. Nine gene promoters were investigated, of which five had already been shown to be promising in our previous study (20), while the other four were known from recent literature to be specifically methylated in cervical cancer (19;21;22). In the present study, only CCNA1 was never methylated in normal cervices and rarely in CIN I. All other genes showed methylation in normal cervices, with CALCA, SPARC and RAR-β2 at high
levels. Methylation frequency of 6 genes (DAPK, APC, TFPI2, SPARC, CCNA1 and CADM1) increased with severity of the underlying cervical lesion. DAPK showed the highest increase in methylation frequency between CIN I and CIN II/III (10% vs. 40% p<0.05), while CCNA1 and TFPI2 were most prominently methylated in cervical cancers compared to CIN II/III (25% vs. 52% p<0.05, 30% vs. 58% p<0.05). CADM1 methylation in cervical cancers was related to depth of invasion (p<0.05) and lymph vascular space involvement (p<0.01), suggesting a role in invasive potential of cervical cancers. Furthermore, in cervical scrapings, CCNA1 was most frequently hypermethylated in cervical cancer cases (80%) and in CIN II/III (56%), while only 25% of CIN I showed methylation. Methylation ratios in scrapings reflected methylation status of the underlying lesions (p<0.05). We concluded from this chapter that methylation of most of the previously reported cervical cancer specific genes also frequently occurs in normal epithelium.

On the other hand, the level as well as frequency of methylation of the various markers increases during cervical carcinogenesis, and CCNA1 and DAPK showed to be the best markers to distinguish normal cervix /CIN I from CIN II/III/cancer lesions. In addition, the number of samples methylated was also increased during carcinogenesis in the corresponding cervical scrapings.

In order to find novel markers that are specifically methylated in all CIN II/III/cervical cancer scrapings, we recently performed a study using pharmacological unmasking of (cervical) cancer cell lines followed by expression microarray analysis in combination with a computational approach. This analysis revealed 45 potentially methylated genes of which 13 gene promoters appeared to be specifically methylated only in cervical cancer and not in normal cervices as shown by BSP (23). In chapter 4, we performed a more in-depth analysis of the methylation patterns of these 13 candidate genes in cervical cancer and normal tissue specimens. In addition, in a large series of scrapings from patients with cervical cancer, CIN I and II/III and from otherwise healthy women, their possible relevance for detection of cervical neoplasia was evaluated. This analysis revealed that 5 of 13 gene promoters (C13ORF18, CCNA1, TFPI2 C1ORF166 and NPTX1) were found to be more frequently methylated in cervical cancer tissues compared to normal cervix specimens. CCNA1, TFPI2 and NPTX1 were previously described to be frequently methylated in cervical cancer (19;22;24). C13ORF18 and C1ORF166 have not previously been described to be methylated in any type of cancer. Quantitative methylation analysis for these 5 markers revealed that CCNA1 and C13ORF18 methylation were both present in 68/97 cervical scrapings from cervical cancer patients and in only 5 and 3 scrapings from 103 healthy controls, respectively (p<0.0005). In cervical scrapings from patients referred with an abnormal PAP smear, CCNA1 and C13ORF18 were methylated in 2/43 and 0/43 CIN 0 and in 1/41 and 0/41 CIN I, respectively. Furthermore, 8/43 CIN II, 22/43 CIN III and 3/3 micro-invasive cancer patients were positive for both markers. Although sensitivity for CIN II or higher (for both markers 37%) was low, specificity (96% and 100%, respectively) and positive predictive value (92% and 100%, respectively) were high. In conclusion, methylation of CCNA1 and C13ORF18 in cervical scrapings is strongly associated with CIN II or higher-grade lesions. Therefore, these markers might be used in a triage setting after primary screening with either
cytology or hr-HPV, in which patients with a methylation positive scraping can be directly referred to the gynecologist for colposcopy and treatment in the same procedure.

Early recurrences after treatment (within 24 months) for high-grade squamous intraepithelial lesions (CIN II/III) might be due to inadequate treatment, while late recurrences (> 24 months) might represent de novo lesions (25). In chapter 5, we used QMSP of 5 gene promoters (DAPK, C13orf18, CADM1, CCNA1 and TFPI2) to determine differences in gene promoter methylation between patients with an early recurrent CIN II/III lesion and a late recurrent CIN II/III lesion and to compare between early and late recurrent lesions the concordance for gene promoter methylation between initial and recurrent CIN II/III lesions. Paired tissue samples were used from 14 initial CIN II/III patients and their recurrent lesions. Our study revealed that early recurrent CIN II/III lesions showed a higher frequency of gene promoter methylation compared to late recurrent CIN II/III lesions in initial lesions (p=0.016) as well as in the recurrent lesions (p=0.032). Except for C13orf18 (k=1.00, p=0.014), in early recurrent CIN II/III lesions, no concordance between gene promoter methylation in initial CIN II/III lesions and early and late recurrent CIN II/III was observed. In conclusion, it seems that frequency of gene promoter methylation is increased in patients with an early recurrent CIN II/III lesion. Therefore, we hypothesize that early recurrence after treatment for CIN II/III lesions may not only be due to inadequate treatment, but also represent a more progressive form of CIN II/III.

Population based screening programs have contributed to a decline of incidence and mortality of cervical cancer. However, in The Netherlands the total non-responders group (people that decide not to participate in population-based screening for cervical cancer) is around 30% which is comparable with other countries. Unfortunately half of the cervical cancers are diagnosed in this group of women (7;26-29). A self-sampling method could increase the participation rate of a non-responder group with 30% (30). Furthermore, self-obtained samples have been shown to be representative by using this self-sampling device for hr-HPV status of the cervix (31). Hence, the combination of a robust methylation marker with a self-sampling device could improve screening for pre-malignant lesions of the cervix dramatically. Chapter 6 describes a feasibility study for the detection of DNA hypermethylation by quantitative methylation specific PCR (QMSP) in cervico-vaginal lavages collected by a novel self-sampling device. Methylation status, cytomorphology and hr-HPV positivity of cervico-vaginal lavages obtained by a novel self-sampling device were analyzed and compared to data obtained from simultaneously collected cervical scrapings from the same 20 patients. This analysis revealed that the concordance between cervical scrapings and lavages for QMSP was high: C13orf18 k=0.600 (p=0.006), CADM1 k=0.583 (p=0.004) and CCNA1 k=0.479 (p=0.024). Comparable concordance was observed for hr-HPV testing between cervical scraping and lavage (k=0.565 (p=0.013)). On the other hand, concordance for cytomorphology between cervical scraping and lavage was low (k=0.273 (p=0.076)). Therefore, we concluded that due to the high concordance of DNA methylation analysis in cervical scrapings and lavages, QMSP in cervico-vaginal samples obtained by a novel self-sampling device has a high potential of detection of malignant
cervical cells. A follow-up study is ongoing to validate the detection of premalignant cervical cells in cervico-vaginal lavages using QMSP.

Future perspectives
In the Netherlands, cervical cancer screening is still based on cytomorphological assessment of cervical smears. Screening is started at the age of 30, and is repeated at 5 year intervals until the age of 60. The new guidelines for cervical cancer screening of the Dutch Pathology Society (NVVP) of 28 June 2006, incorporated the use of hr-HPV DNA testing in the triage of women with borderline cytological abnormalities (Pap2/Pap3A1), and proposed a follow-up flow-chart based on the results of a study by Bais et al. (32). In the new guidelines, in all women with Pap2/Pap3A1, cytology and HPV test is repeated after 6 months. Women with Pap2/Pap3A1 remain in the screening program without referral to a gynaecologist if no hr-HPV infection is detected; in these women, cytology and HPV test are repeated once more after 12 months. Hr-HPV positive women with Pap2/Pap3A1 will be referred to a gynaecologist for colposcopy. Women who have cleared hr-HPV infection are no longer at risk for development of premalignant lesion. Further surveillance can take place within the screening program at the normal interval of 5 years.

Cervical cancer is believed to have a co-facto rial etiology in which HPV infections are considered the most important factor (33). The risk of acquiring a HPV infection is highly associated with early sexarche and sexual contact with promiscuous partners. The cofactors, i.e. long-term oral contraceptive use, high parity, other sexually transmitted infections, cigarette smoking, and viral cofactors as viral load, integration, genotype and variants influence the likelihood of HPV persistence and progression towards a (pre-)malignant cervical lesion (34). HPV DNA can be detected in cytological smears and histological samples by a number of methods that have been developed over the past 25 years. Amongst these techniques are a variety of target-amplification systems like type-specific PCR and consensus-primer PCR and signal-amplified, immunoassay-based nucleic acid hybridization methods such as the Hybrid Capture assay. The sensitivity of hr-HPV testing for the detection of cervical cancer and CIN II/III lesions is approximately 95% (35;36). This by far exceeds the sensitivity of conventional smears and liquid based cytology, ranging from 30% to 87% for both methods (37). However, the specificity of a positive hr-HPV DNA testing for cervical cancer and CIN II/III lesions is still lower than that of both conventional and liquid based cytology. There is therefore ample room for new biomarkers for cervical neoplasia to be explored.

Since cervical cancer develops from a distinct premalignant stage, the definition of a perfect methylation marker in cervical cancer implies a marker that is always methylated in cervical cancer and CIN II/III lesions and never methylated in CIN I and controls. In chapter 3, we analyzed the methylation status of 9 gene promoters in patients with different grades of cervical neoplasia. The methylation level of all gene promoters increased with severity of the underlying lesion, but 8 of 9 gene promoters also showed some methylation in controls (normal cervices).
In order to implement methylation analysis in a diagnostic setting, preferably, one should aim to identify a marker that is always negative in normal cervices in such a way that still many cancers would be positive. Only CCNA1 and C13orf18 met these criteria (see chapter 4). Both gene promoters showed potential to become a diagnostic biomarker for CIN II/III and cancer in a large series of scrapings. Targeting both as a single gene promoter, 70% of cervical cancers was detected and around 40% of CIN II/III lesions. Almost none of the CIN 0 and CIN I were positive, resulting in a high specificity and positive predictive value. The methylation pattern of these two genes was quite similar in cervical neoplasia as almost all the same lesions were positive for both gene promoters. The diagnostic performance of a “methylation test” therefore did not improve by combining the two genes in one test. In colorectal cancer, Frigola et al. found DNA hypermethylation within the repressed genomic neighborhood, localized to three separate enriched CpG island ‘suburbs’, with the largest hypermethylated suburb spanning 1 Mb (38). As CCNA1 and C13orf18 are both located on chromosome 13 and close to each other, the same mechanism might exist in cervical cancer. However, preliminary data from our group showed for a selected group of gene promoters located in the region between CCNA1 and C13orf18 no methylation in cervical cancer cell lines. Another mechanism of methylation in cervical cancer therefore might play a role or it is just coincidence that these 2 gene promoters show the similar methylation pattern. If it is still regional loss of chromosomes, the region between CCNA1 and C13orf18 might not only be inactivated by promoter hypermethylation, but also deregulation of histone deacetylation or nucleosome remodeling might be important.

In order to reach a methylation-based test with theoretically 100% sensitivity for cervical cancer detection, we are currently looking for new hypermethylated genes located in other chromosomal regions. These new genes should fill the gap and an ideal gene panel will be set up finally. Novel genome-wide methodology is used in order to search for CIN II/III/cervical cancer specific methylation markers such as Methylated DNA Immunoprecipitation (MeDIP) (39) in combination with methylation-specific oligonucleotide microarray (tiling array) (39;40) or sequencing (41) and Illumina GoldenGate® Methylation (see Chapter 2). By MeDIP, methylated DNA is enriched by immunoprecipitation of 5mC residues (39). In line with the usage of MeDIP, one could also purify methylated DNA by the usage of antibody-coupled methyl-binding domain (MBD) proteins (i.e. chromatin immunoprecipitation (ChiP)) (42-44). Advantages of MeDIP/ChiP are the small amount of DNA necessary to perform these experiments and the usage of archival paraffin embedded tissue as the DNA used as input for MeDIP/ChiP should be in the range of 300–1000 bp, which enables retrospective studies. For high-throughput sequencing, a number of technologies are currently available, including pyrosequencing (Roche/454), sequencing by ligation (ABI SOLiD), and reversible terminator sequencing (Illumina/Solexa). These technologies allow to quickly and inexpensively sequence very large amounts of DNA but all have the drawback of generating short (≥400 nt) or very short (36–50 nt) sequence reads. These next-generation sequencing techniques can be used in tandem to provide independent methods for validation of whole genome methylation profiling.
Until now, none of these approaches has been used in the search for cervical cancer specific methylation markers.

Prophylactic HPV vaccines are currently approved by the FDA and will be introduced in nationwide vaccination programs (45-48). In 20 years from now, this will most likely result in a lower prevalence of cervical cancer and its precursor lesions, thereby increasing the likelihood of false positive screening results. Detection of remaining cervical neoplasia will therefore require screening tools with a higher specificity. QMSP has the potential to become the new screening tool, depending on the new methylated genes to be discovered. Due to the current lower sensitivity compared to hr-HPV testing, the road to clinical application of QMSP is still very long. With discovering new gene promoters specifically methylated in cervical cancer and CIN II/III lesions QMSP will be more sensitive, while maintaining its high specificity.
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