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Review

An overview of innovative techniques to improve cervical cancer screening

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Abstract. Although current cytomorphology-based cervical cancer screening has reduced the incidence of cervical cancer, Pap-smears are associated with high false positive and false negative rates. This has spurred the search for new technologies to improve current screening. New methodologies are automation of Pap-smear analysis, addition of new biological or molecular markers to traditional cytology or using these new markers to replace the current screening method. In this overview we will summarize data on cervical cancer epidemiology and etiology and the current cervical cancer screening approach. Available data on new screening approaches, such as quantitative cytochemistry, detection of loss of heterozygosity (LOH) and hypermethylation analysis will be reviewed. We discuss the potential of these approaches to replace or augment current screening. When available, data on cost–effectiveness of certain approaches will be provided. In short, Human Papillomavirus (HPV) DNA detection stands closest to implementation in nation-wide screening programs of all markers reviewed. However, specificity is low in women aged <35 years and the psychological effects of knowledge of HPV positivity in absence of cervical (pre) malignant disease are important drawbacks. In our opinion the results of large clinical trials should be awaited before proceeding to implement HPV DNA detection. New technologies based on molecular changes associated with cervical carcinogenesis might result in comparable sensitivity, but improved specificity. Hypermethylation analysis is likely to be more objective to identify patients with high grade squamous intra-epithelial lesions (HSIL) or invasive cancer with a higher specificity than current cytomorphology based screening.

Keywords: Biological marker, HPV, methylation, cervical cancer, cervical intraepithelial neoplasia, screening

1. Introduction

Cervical cancer is an important cause of death worldwide. Persistent infection with human papillomavirus (HPV) plays an essential role in cervical carcinogenesis. Current cervical cancer screening is performed by cytomorphological assessment of cervical smears. Because of high false positive and false negative rates, efforts have been made to improve cervical cancer screening by the use of HPV DNA testing or other new biological and molecular markers. In this overview we will summarize data on cervical cancer epidemiology and etiology and the current cervical cancer screening approach. Molecular diagnostic targets such as microsatellite alterations, telomerase activity and gene promoter hypermethylation for cervical cancer detection will be reviewed in more detail.

1.1. Epidemiology and etiology of cervical cancer

The cumulative lifetime risk for a woman to develop cervical cancer varies from 0.4% in Israel to 5.3% in Colombia. Cervical cancer represents the sec-

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ond most frequent gynecological malignancy among women worldwide, with the highest incidence rates in less developed countries [97]. It was estimated that 10,370 new cervical cancer cases were to be diagnosed in 2005 in the US and an estimated 3710 deaths from cervical cancer would occur (accounting for 1.6% of all cancers and 1.3% of all cancer related deaths in women) [57]. In sharp contrast to these relatively low incidence numbers of cervical cancer are the high number of surgical treatments each year for pre-malignant cervical lesions in countries with nation-wide screening programs [64,94,130]. Cervical cancer develops from these pre-malignant lesions, also called cervical intraepithelial neoplasia (CIN). The mildest form, CIN I, regresses in most cases, while 20–45% of the CIN II/III lesions will progress to cervical cancer when left untreated [98]. It is estimated that the progression from CIN to cervical cancer generally takes 10–15 years [82].

Sufficient evidence for a causal role of HPV in cervical carcinogenesis has been provided by both epidemiological as well as experimental studies in different parts of the world. It has been proposed that HPV infection is the first identified necessary cause of cervical cancer, implying that cervical cancer, with very rare exceptions, cannot develop without HPV infection [14, 131,147]. Over 100 HPV types have been identified of which more than 35 types can be found in the genital tract and 18 are associated with cervical carcinogenesis [85]. Expression of the viral proteins E6 and E7 is pivotal for cervical carcinogenesis because E6 and E7 facilitate increased degradation of two important cellular regulatory proteins, p53 or pRB, respectively [45]. HPV 16 accounts for 46–63% of the cervical cancer cases in most countries [26]. The second most frequent HPV type is HPV 18 (10–14%) followed by HPV 45 (2–8%) and 31 (2–7%) [26]. Despite of all the evidence for the important role of HPV in cervical carcinogenesis it is clear that additional factors, both viral and host-cell related, have to be involved because the majority of patients infected with HPV will not develop invasive cervical cancer [7,147].

1.2. Current cervical cancer screening

Cytomorphological examination of cervical smears is the most widely applied screening-method for cervical cancer and its precursors. In 1941, it became clear that cytomorphological assessment of cervical smears could be used to detect cervical cancer and its precursors [96] and many countries started to organize screening programs. Cervical smears are classified according to a modified Papanicolaou system (Pap/CISOE-A) or the Bethesda classification system. An overview of the different nomenclature used in cervical cytomorphology and histomorphology is given in Table 1.

Although the introduction of nation-wide screening programs have led to decreasing incidences of cervical cancer it has been questioned whether the disadvantages counter-balance the relatively low reduction in cervical cancer deaths [100,101]. However, a more recent evaluation of cervical cancer screening in Britain suggests that introduction of a national screening program has prevented an epidemic of cervical cancer deaths [98]. Disadvantages of the current screening method include the high numbers of false-positive and false-negative cervical smears, leading to an overshoot of diagnostic procedures or even a delay in the diagnosis of cervical cancer [64,71,94]. Up to 14% of all cervical smears are cytomorphologically abnormal without the presence of a (pre)malignant cervical lesion [6]. These false-positive results can cause unnecessary anxiety and invasive procedures. False-negative cytology may be found in about 50% of cases when previous negative smears are reviewed from the small propor-

Table 1
Cytomorphological and histomorphological nomenclature

Dysplasia	CIN	Bethesda	Papanicolaou
Normal	Normal	Within normal limits	Pap 1
Benign atypia	Inflammatory atypia	Benign cellular changes	Pap 1
Atypical cells	Squamous atypia	ASCUS	Pap 2
Mild dysplasia	CIN I	Low-grade SIL	Pap 3A1
Moderate dysplasia	CIN II	High-grade SIL	Pap 3A2
Severe dysplasia	CIN III	High-grade SIL	Pap 3B
Carcinoma *in situ*	CIN III	High-grade SIL	Pap 4
(Microinvasive) cancer	(Microinvasive) cancer	(Microinvasive) cancer	Pap 5

ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; SIL, squamous intraepithelial lesion.

tion of screened women who develop invasive cancer [65,106]. High grade CIN or micro-invasive cervical cancer has cure rates close to 100% with appropriate treatment. False-negative screening results will leave CIN or cancer undetected. Even when symptoms start to occur, the assurance given by a false negative smear may lead to a further delay in the diagnosis and treatment of the cancer which will negatively influence curation chances.

Low attendance to the screening programs is another major drawback of cervical cancer screening. One of the reasons is that many women feel embarrassed to undergo a vaginal examination. Half of cervical cancer cases arise in women who are not adequately screened [107,126]. Therefore, it has been investigated whether self-sampling can overcome this problem and whether it is a reliable alternative to physician-collected samples [28,90]. Although feasible, patient-collection seems to be an inferior alternative to physician-collected cervical cytology [32,40, 90].

2. Technical improvements for morphological screening

2.1. *Automated Pap-smear analysis*

For automated slide microscopy digital photographs are taken of ordinary Pap-smears. The data thus obtained are documented and images can be analyzed by computer systems such as done by the neural-network-based approach and by the AutoPap system [27,78, 137,139]. These automated methods have the potential to improve the accuracy of cervical cytological examinations and can result in a higher productivity of cytology laboratory personnel [137]. For the detection of high-grade cervical neoplasia (CIN II/III or HSIL and cervical cancer) in a primary screening setting, both systems show slightly improved specificity and equivalent sensitivity to the Pap test [27,78,137]. Confortini et al. therefore stated that comparison of the AutoPap system and conventional reading should focus mainly on cost analysis [27]. However, the current expense of the automated technologies in comparison to conventional screening limit their widespread implementation [44]. At present it is not clear whether the potential benefits of computerized screening relative to conventional Pap-smears are sufficient to justify a possible increase in costs [124,125]. Nonetheless, in the future widespread implementation of automated Pap-smear may become cost-effective since costs of manpower will further increase, whereas automated technologies in general become less expensive in time.

2.2. *Liquid based cytology*

For liquid based cytology, also called thin-layer test or ThinPrep Pap test, cervical cells are collected by scraping the cervix with a sampling device made of plastic, such as the “broom-like” cervix brush. This device is then transferred directly to a vial containing a liquid preservative. Collected cells are mechanically dispersed into the liquid medium before a representative aliquot is transferred to the slide as a monolayer. This in contrast to conventional Pap-smears, where the cervix is scraped with an Ayers spatula and/or cytobrush and the collected material is then directly smeared on a glass slide and fixated with a fixative. Possible advantages of this procedure compared to conventional Pap-smear testing are (1) prevention of inadequate air drying of cells after sampling and (2) removal of debris and blood before cell transfer to slides and enhancement of cellular separation. Furthermore, several slides can be tested from one sample and the residual fluid can be used for a variety of other (molecular) analyses. Importantly, all aliquots from the same collected medium will be similar in composition.

Bishop et al. compared conventional Pap-smear classification with classifications obtained by liquid based cytology and showed a 97% correspondence within one diagnostic category [12]. In another study 8636 Costa Rican women were tested. Sensitivity for CIN II or worse was 99% for both conventional and liquid based cytology, whereas the specificity of smears with at least moderate dyskaryosis for histological CIN II or worse was 64% for conventional cytology and 69% for liquid based cytology [54]. In a multi-center screening study, liquid based cytology showed a 58% reduction in unsatisfactory slides [38]. Most studies report that liquid based cytology provides more satisfactory results and has slightly higher sensitivity for high-grade cervical neoplasia compared to conventional Pap-smears [5,10,12,74–76,95,99,135]. However, one large randomized trial in 1,999 women showed that the quality of conventional Pap-smears sampled after removal of mucus and cellular debris with a cellulose swab was better than that of liquid based cytology [93]. Correlation between cytological and histological diagnoses was also better for conventional Pap-smears than for liquid based cytology, whereas for the detection of adenocarcinoma *in situ*, liquid based cytology performed better [4]. In a recent meta-analysis by Davey et al. no difference was found between liquid-based and conventional cytology in the percentage of unsatisfactory slides when all studies were compared, while

in higher quality studies no evidence was found that liquid based cytology is more accurate in detecting HSIL [29]. The use of liquid based cytology is associated with an increase in costs of cervical cancer screening because the average cost of a thin-layer test is $2.4 higher than that of the conventional smear test [81]. The higher price of liquid based cytology is only justifiable if this screening technique outperforms the conventional method [81]. Current evidence appears to be insufficient to recommend for or against the routine use of liquid-based cytology especially because it is unclear whether its use will reduce the incidence of and mortality from invasive cervical cancer despite the reported increase in sensitivity [124,125]. The guideline of the American Cancer Society for the early detection of cervical neoplasia and cancer states that cervical screening using liquid-based cytology may be an alternative to conventional screening [108]. In our opinion efforts should be made to develop a less expensive way to perform liquid-based cytology by comparing the commercially available cell preservatives with alternative preservatives, for example ethanol–carbowax (7% polyethylene-glycol, 50% ethanol). If the average costs of a thin-layer test would be comparable to the average costs of a conventional Pap-smear, the integration of liquid based cytology in existing screening programs would be justifiable, and we could benefit from the improved sensitivity and the number of satisfactory slides. Finally, as discussed previously, when new screening methods based on the presence of biological and molecular markers, such as HPV DNA analysis or gene promoter hypermethylation analysis will be introduced in cervical cancer screening, the residual fluid can be used for detection of such markers in addition to liquid based cytology.

3. New markers in cervical cancer screening

Improvement of specificity of cervical cancer screening would lead to the reduction of costs associated with false positive results. Improvement of sensitivity could result in a higher detection percentage of premalignant or early stage malignant cervical lesions. In the following parts of this review we describe whether molecular markers may be able to improve cervical cancer screening.

Staining of biological and molecular markers superimposed over the conventional Pap-smear

3.1. Quantitative cytochemistry

Immunocytochemical staining of markers of cell proliferation

We will first discuss markers that can be used superimposed over the conventional Pap-smear. These markers exploit the advantage of both Pap-smear analysis and the molecular identification of dysplastic cells. Dysplastic cells can be recognized by their increased persistence in cell cycle compared with normal epithelial cells that exit the cell cycle during maturation and differentiation [138]. Ki-67 antigen is a proliferation marker, which is expressed during late-G1, S and G2M phases of the cell cycle, but not during G0 [21,41]. Immunocytochemical staining of Ki-67 antigen by the MIB1 antibody is strongly related to the severity of cervical neoplasia [1,20,80] and also to HPV infection [1,43,48]. Furthermore, a model based on Ki-67 staining can predict which low grade squamous intra-epithelial lesions (LSILs) are likely to progress and Ki-67 staining was also proposed as a triage tool for patients with minor Pap-smear abnormalities [31,67]. The immunocytochemical staining of PCNA (the proliferating cell nuclear antigen), a protein essential for the synthesis of DNA during cell proliferation, was associated with grade of SIL [114] and positivity was significantly related to increased severity of CIN [42]. Ki-67 and PCNA staining on Pap-smears can be used to distinguish high-grade CIN lesions from atrophic cervical epithelium [18,19,34,42, 127]. PCNA staining has not been evaluated for possible use in primary cervical cancer screening. A recent estimation of the cost–effectiveness of the use of Ki-67 revealed that Ki-67 quantification superimposed over Pap-smears would prevent 8250 of 66,000 (12.5%) patients with CIN $\leqslant$ II from being over treated annually in Europe [2]. In two studies, both Ki-67 and PCNA staining were compared with two other markers (Cdc6 and Mcm5) that are involved in the initiation of DNA replication. Higher percentages of dysplastic cells in high and low-grade squamous intraepithelial lesions were immunocytochemically stained by both Cdc6 and Mcm5 compared to Ki-67 and PCNA staining [138]. In addition, when Cdc6 and Mcm5 were superimposed over Pap-smears, their expression was highly associated with severity of CIN lesion [87,138]. Furthermore, the correlation between Mcm5 staining intensity and HSIL was independent of HPV status, and could therefore be a potential biomarker in both HPV positive and negative cervical dysplasia [87]. However, these markers of DNA replication may also detect im-

mature phases of squamous metaplasia which can lead to false positive results [138]. The data on Cdc6 and Mcm5 immunocytochemistry need to be further evaluated in larger studies comprising pre-clinical screening populations. $P16^{INK4A}$ is a cyclin dependent kinase inhibitor, which accumulates in cells infected with HPV. The drawback of current cytological screening is that it is difficult to localize abnormal cells amidst an abundance of normal cells. In several studies immunostaining of $P16^{INK4A}$ has been investigated as a tool to identify the dysplastic cells [62,86,87,134]. These studies suggest that immunostaining of $P16^{INK4A}$ of cervical scrapings improves the interpretation of the degree of abnormality of the slides. It has been shown that staining for $P16^{INK4A}$ improves inter-observer agreement and it has been proposed as a method to improve the quality of screening [62,63].

3.2. *Markers for DNA aneuploidy*

Other markers of dysplasia to be detected in the same smears as used for morphological Pap-smear analysis are markers to analyze DNA aneuploidy. In cervical smears and tissue samples, aneuploidy was related to the severity of neoplasia using Feulgen-stained image analysis [11,83]. Monsonego and coworkers found aneuploidy to be present in 78% of smears taken from patients with high-grade CIN and in 21% of smears taken from CIN I patients. Furthermore, it has been suggested that ASCUS cases (atypical squamous cells of undetermined significance) with rare aneuploid cells and positivity for high risk HPV represent lesions that are biologically similar to HSIL and are likely to progress [13]. In a more recent study, Nguyen et al. found aneuploidy in cervical smears of 67 of 72 (93%) patients with CIN III, 26 of 31 (83%) patients with CIN II, but also in 12 of 18 (67%) patients with histologically confirmed CIN I. The inter-observer reproducibility of DNA-image cytometry was very good ($\kappa = 0.87$) [89,119].

In summary, both aneuploidy measurements and immunocytochemistry for Ki-67, $p16^{INK4A}$, PCNA, Mcm5 and Cdc6 proteins should preferably be used superimposed over conventional Pap-smear analysis, which will lead to the development of a labor intensive and therefore expensive screening approach. New PCR-based detection techniques may be used without concurrent morphological screening and may therefore provide a more clear-cut approach.

4. Detection of molecular markers by PCR-based techniques

4.1. *Human papillomavirus and cervical cancer screening*

HPV plays an important causative role in cervical carcinogenesis. It is estimated that for women the lifetime risk of contracting a genital HPV infection is 80%, leading to genital warts in 5%, abnormal cervical scrapings in 35%, CIN in 25% and invasive cervical cancer in less than 1% [66,110,120,143]. HPV can be detected in almost all HSIL lesions and cervical cancers and can be detected in cervical smears as well [15]. It has therefore been suggested that the detection of high-risk HPV in cervical smears may well improve cervical cancer screening because of high sensitivity [16,113]. Over 100 studies were undertaken to proof this suggestion. In general, the sensitivity of high-risk HPV testing by general primer PCR (GP5+/6+; MY 09/11; CP1/2G; SPF10) or Hybrid Capture II (HC II) to detect HSIL and cervical cancer is more than 90%. However, women without cervical dysplasia can also be HPV positive. The percentage of women that are HPV positive but have a cytologically and/or histologically confirmed normal cervix varies from 5% in Europe to 26% in Sub-Saharan Africa [25]. Therefore, due to low specificity, HPV testing alone will not sufficiently improve cervical cancer screening [6,85]. Still, HPV testing may be used in specified patient groups [109]. Testing of HPV might improve cervical cancer screening in women with smears showing borderline abnormalities (borderline dysplasia or atypical squamous/glandular cells of undetermined significance (ASCUS/AGUS)), because it could prevent a number of referrals. Women with ASCUS who are HPV negative do not need referral because these women very rarely will have HSIL or cervical cancer. In women with ASCUS, HPV testing will identify more patients with high-grade neoplasia compared to repeat cytology. However, a specificity of 59–64% in this group of patients will still lead to more referrals than necessary [112]. HPV testing may also be used together with cytology to define a patient group in which screening intervals can be prolonged. HPV negative women with normal cytology have an extremely low risk to develop cervical cancer in 10 or more years [14,60]. Other patients that may benefit from HPV testing are women aged 35 years or older. In these women HPV testing has a higher specificity because HPV infections are less frequent and when

present, infections more often represent HPV persistence [24,61]. In women with cytological abnormalities HPV persistence is strongly related to the development of HSIL and cervical cancer [91]. In the US the HC II test, that can detect 13 different oncogenic HPV types, has recently been approved for screening of women of 30 years and older in addition to cytology, and for triage of women with ASCUS/AGUS. The benefits and costs of adding the HPV test to conventional cytology have been investigated by comparing 18 different screening strategies [77]. The HPV test alone was equally effective compared to cytology alone, at any screening interval, but with increased costs. Addition of the HPV test to cytology, at an interval of 2 years and commencing screening at age 20 saved the maximum number of lives, with an incremental cost of $76,183 per quality adjusted life year. This was considered to be cost-effective by Mandelblatt et al., however, applying age limits for addition of HPV test to conventional screening might maintain the number of lives saved while reducing costs [77]. A more recent study, analyzing data from two large clinical trials, has shown that testing for more than 10 HPV types decreased specificity more than it increased sensitivity [109].

Resuming, HPV testing can be used as a diagnostic tool for screening purposes, but it has a relatively low specificity due to frequent HPV infections without dysplasia. HPV DNA testing can best be restricted to a subset of females in which specificity of HPV testing is higher. Even though in the US the HCII test is already used combined with cytology in the cases previously mentioned, the definitive evidence of efficacy from long-term follow-up studies and from randomized trials should best be awaited before considering implementation of HPV DNA testing in existing screening programs. Trials are underway that should soon clarify the role of HPV testing cervical cancer screening [17,125].

The psychological consequence of a positive high-risk HPV test without the presence of HSIL or cancer will be considerable. Regardless of how it is implemented, the incorporation of HPV DNA testing into primary screening will inevitably result in informing millions of women with normal Pap-smears world wide that they are at increased risk for cervical cancer [142]. Current evidence shows that women are ill informed about the relationship between HPV, the sexually transmitted nature of HPV and cervical cancer and suggests a role for clinicians to educate women so that implementation of HPV DNA testing in current successful screening programs will not reduce participation of women by causing anxiety and stigmatization [3,132]. It will be necessary to assure HPV positive women that they do not have to feel unduly alarmed or stigmatized while convincing them of the need for proper follow-up in order to identify those who will actually develop cervical cancer.

In light of these considerations, the detection of other markers of molecular changes occurring in cervical carcinogenesis should also be investigated. Most of the molecular markers and assays in the following parts of this review have only recently been validated in cervical cancer. For most of these new techniques it will be difficult to estimate possible cost–effectiveness which will therefore not be discussed.

4.2. Loss of heterozygosity

Since HPV infection does not always lead to cervical cancer, other genetic alterations must also play a role in tumor-development. Oncogenes and tumor suppressor genes are genes involved in carcinogenesis. Oncogenes may foster malignant processes if activated, whereas tumor suppressor genes, because of their repressive function on e.g. cell cycle progression or DNA repair, usually will be inactivated during malignant transformation. Possible mechanisms to downregulate tumor suppressor genes are point mutations, DNA methylation of the promoter sequences or deletions of (parts of the) tumor suppressor genes. To inactivate tumor suppressor genes located on autosomal chromosomes both alleles need to be inactivated, often by combinations of different mechanisms [146]. For instance, the loss of one of the alleles can be detected quite easily by the analysis of loss of heterozygosity (LOH). To determine LOH, DNA from tumor tissue is compared with normal DNA of the same individual using PCR-based microsatellite analysis. Microsatellites are short tandem repeats of variable lengths of di-, tri- or tetranucleotides that are randomly distributed throughout the human genome. Many microsatellite markers are highly polymorphic and can therefore be used to discriminate between the two alleles. In case of a deletion, the intensity of one of the alleles in the tumor sample will decrease compared to the ratio of the alleles in the normal DNA. In cervical neoplasia, frequent LOH has been found on several chromosome arms, including 1, 3p, 4p, 4q, 5p, 6p, 9p, 11p, 11q and 17p [23,73]. However, the putative tumor sup-

pressor genes located on these chromosomal locations are yet to be identified. The frequency of LOH in premalignant lesions increases with severity [72] and is related to presence of HPV DNA [35]. Using DNA from Pap-smears and 9 microsatellite markers, LOH was present in 85% of 13 smears taken from patients with cervical cancer [105]. For the reliable detection of LOH, (1) a sample of normal tissue from the individual is needed to compare the DNA to the tumor sample and (2) more than 50% of the cells in sample have to be tumor-derived. For this reason, LOH analysis is not suitable for cervical cancer screening by analysis of cervical scrapings [115].

4.3. Telomerase activity

Telomerase is a nuclear enzyme that is able to synthesize short stretches of repeat nucleotides that are lost from telomeric ends of chromosomes with each round of replication. Studies in cancer cell lines as well as in human tumors showed that, in contrast to normal somatic cells, the vast majority of malignant cells (>90%) are characterized by increased telomerase activity [68,102,111]. Therefore, determination of telomerase activity was suggested for early cervical cancer detection. Telomerase activity is associated with severity of cervical neoplasia [48,55,140,145]. The percentage of telomerase activity in cervical cancer tissue varies from 64% to 97% [69,92,102,140]. However, in cervical scrapings positivity for telomerase activity was found in only 31% of cervical cancer patients [140]. For CIN lesions percentages of telomerase activity are reported between 0 and 68% [56, 69,102,103,116,140]. Ngan et al. reported that telomerase activity assessment was unable to improve the detection of high-grade CIN in a study in 86 women with normal cytology and 114 women with abnormal cervical smears [88] and this was confirmed by Jarboe et al. [56]. Testing for both telomerase activity and the telomerase components hTR and hTERT in cervical scrapings led to the conclusion that detection of telomerase activity and components are not suitable for the detection of CIN II or more severe lesions in women with cytological borderline, mild or moderate dyskaryosis. Furthermore, the combined sensitivity and specificity of these tests were too poor to suggest a role in primary screening [103]. In summary, it can be concluded that telomerase analysis is not suitable for cervical cancer screening.

4.4. DNA methylation

Some 30 years ago it became clear that, as a “fifth base”, methylcytosine is formed post-replicatively in DNA by addition of a methyl group to a cytosine already incorporated into previously synthesized DNA [133]. Methylation forms a modification of DNA and is referred to as an epigenetic change since it does not alter the primary DNA sequence. The function of DNA methylation may be a contribution to overall genetic stability and maintenance of chromosomal integrity and to facilitate organization of the genome into active and inactive regions with respect to gene transcription [9,22,49].

Genes with CpG islands in the promoter region are unmethylated in normal tissues. Exceptions are inactivated genes on the female X-chromosome and the inactivated allele for selected imprinted genes on autosomal chromosomes. For the past decade, abnormal patterns of DNA methylation have been recognized as molecular changes in neoplasia [59]. The CpG islands in promoter regions of genes are targets for methylation and upon hypermethylation, transcription of the affected genes may be blocked, resulting in “silencing” of these genes. Hypermethylation 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. Hypermethylation is suggested to be an early event in carcinogenesis of various tumor types [8].

The detection of DNA methylation as novel biomarker in cancer research and diagnostics was revolutionized by two major discoveries. The first was the discovery of a very simple assay to visualize methylcytosine by treatment of genomic DNA with sodium bisulfite. Bisulfite treatment results in the conversion of cytosine residues into uracil, except methylcytosine residues that are protected against this treatment. In this way, by the use of sequence analysis methylated and unmethylated DNA can be distinguished [39]. The second discovery was the development of methylation specific PCR. By taking advantage of the sequence differences within CpG islands of a promoter after bisulfite treatment, specific PCR primers can be designed that can distinguish methylated DNA from unmethylated DNA [50]. From that time on, numerous different assays have been developed and used to study methylation based on the methods as reviewed by Laird [70]. DNA methylation analysis has been used to identify new biomarkers in various tumor types [36,58,79].

Promoter hypermethylation analysis has also resulted in the identification of a number of biomarkers specifically hypermethylated in cervical cancer. Promoter hypermethylation of at least one of the genes *p16*, *DAPK*, *MGMT*, *APC*, *HIC-1* and *E-cadherin* was present in 79% of 53 cervical cancers and in none of 24 normal cervical tissues [30]. Furthermore, promoter hypermethylation of at least one of the genes *p16*, *RARβ*, *FHIT*, *GSTP1*, *MGMT* and *hMLH1* was detected in 14 of 19 cervical cancers; in 12 of 17 HSIL's; in 11 of 37 women with no dysplasia or with CIN I and in none of 22 negative control tissues [129]. Other more recent potentially promising gene promoters that are hypermethylated in cervical cancer are *TSLC1* and *TWIST1*. *TSLC1* hypermethylation was observed in 59% of 49 cancers, 35% of 20 HSIL's and in none of 11 LSIL's [118]. Aberrant methylation was observed in 68 of 92 (74%) squamous cell cervical cancers and 13 of 23 (57%) CIN III/CIS for at least one of the genes *DAPK*, *RARβ* and *TWIST1*. *TWIST1* was aberrantly methylated in 24 of 56 (43%) cervical cancers and in 3 of 13 (23%) HSIL's. As a diagnostic test MSP of these three genes would result in a specificity of 95% and a sensitivity of 74% [37].

All these experiments were carried out using conventional methylation specific PCR (MSP). An advancement of conventional MSP is real-time quantitative methylation specific PCR (QMSP) which permits quantification of methylated DNA after adjusting for DNA input. It is more sensitive than conventional PCR and can detect aberrant methylation patterns in samples with substantial contamination of normal DNA (1 : 10.000) [33]. Furthermore, it is a high-throughput technique, which can facilitate the implementation of QMSP in nation-wide screening programs. It has been demonstrated by QMSP and MSP analysis that cervical scrapings reflect the hypermethylation status of the underlying cervical epithelium well [37,104]. Using QMSP, we have previously demonstrated that 32 of 48 (67%) cervical carcinomas were hypermethylated for at least one of the *APC*, *DAPK*, *GSTP1* and *MGMT* genes in cervical scrapings [104]. In a follow-up study we performed QMSP with 12 previously identified cancer-related methylation markers (including *APC* and *DAPK*) on cervical scrapings from 19 healthy controls and 28 cervical cancer patients to determine specificity and sensitivity. This analysis revealed that hypermethylation of any of a combination of 4 genes resulted in a sensitivity of 89.3% and specificity of 100% [141]. New methylation markers specific for cervical cancer need to be added to the 4 selected markers to increase sensitivity. One approach to find such markers is to analyze many more reported methylation markers and perform hierarchical clustering analysis [84]. Another approach for new marker discovery is the use of high throughput screening techniques such as expression-microarrays to find cancer-specific downregulated genes in combination with algorithms that predict methylation sensitive genes. Such an approach was reported for the identification of methylation markers in head and neck- and esophagus cancer [122,144], and recently also for cervical cancer, measuring upregulation in cervical cancer cell lines after treatment with the demethylating agent DAC [117]. The 23 upregulated genes that were identified were validated for hypermethylation in cervical scrapings from 21 normals and 22 invasive cervical cancers in a semi-quantitative manner. This resulted in nine genes that were found to be hypermethylated in cervical cancer. Six of these genes were further analyzed using QMSP. The most promising genes were SPARC and TFPI2, which were methylated in 20 of 22 cervical cancer scrapings and in only 3 of 21 normal scrapings. This is the highest level of methylation for a single gene thus reported [117].

In summary, detection of (pre)malignant cervical neoplasia based on changes in DNA hypermethylation occurring during cervical carcinogenesis appears to be appealing. Many hypermethylation markers have been reported already as possible markers in the diagnosis of various (pre)cancers and other diseases [51,52,117, 123,136]. At present, the first diagnostic tests based on promoter hypermethylation are becoming available such as *MGMT* in glioblastoma [47] and *GSTP1* in prostate cancer [121]. A screening test based on hypermethylation detection is not yet available for cervical cancer. To date, no estimates have been made on the cost-effectiveness of a screening test based on hypermethylation analysis. However, if future studies reveal that QMSP can be used as a replacement of the current approach, QMSP alone is likely to be less labor intensive than cytomorphology based screening and might therefore be cost-effective. If QMSP will be used in addition to the current approach it is likely that this will not be cost-effective.

5. Conclusion and future perspectives

Current morphology based cervical cancer screening is associated with significant false positive and negative results. In this review we showed that at present

no other diagnostic tools are available with proven cost–effectiveness to replace or augment current Pap-smear screening. HPV DNA detection stands closest to implementation in nation-wide screening programs of all markers reviewed. However, low specificity for (progressive) high-grade CIN and cervical cancer in women aged <35 years and negative psychological effects of knowledge of HPV positivity are important drawbacks. Even when the trials that are underway would show cost-effectiveness of implementing HPV DNA testing in cervical cancer, screening with new markers and technologies could theoretically still lead to major improvements. Implementation of liquid based cytology is not likely to improve Pap-smear screening. However, it would aid the development of new technologies which can be tested in residual fluid and the results thus obtained can be compared with the paired morphologically classified cytology.

It appears that, of the more experimental biological and molecular markers, promoter hypermethylation analysis holds most potential as a new diagnostic test for cervical cancer. In comparison to morphology based Pap-smear analysis QMSP has three major theoretical advantages: (1) clear-cut results after the definition of CIN II/III and cervical cancer cut-off levels; (2) QMSP is amenable to high-throughput analysis; and (3) promise of high sensitivity combined with high specificity. When a gene panel will be discovered with sensitivity and specificity that exceed conventional cytology and equals the combination of cytology and HPV DNA analysis, QMSP can be used to replace the current screening techniques. Although current results for hypermethylation analysis are promising, more studies including larger patient populations, preferably randomized clinical trials, need to be performed.

Primary prevention of cervical cancer by effective prophylactic HPV vaccines may be possible in the near future which will further challenge cervical cancer screening. Results of vaccination trials are promising, with seroconversion observed in almost all vaccinated subjects, and no CIN lesions observed in the vaccinated groups [46,66,128]. HPV vaccines have recently been approved by the Food and Drug Administration, but how HPV vaccination will be implemented is still subject to discussion. Based on a mathematical model described by Hughes et al. assuming a 90% vaccine coverage, a 75% effectiveness and an immunity that lasts 10 years, vaccinating both men and women would reduce the prevalence of HPV with 44% [53]. Vaccinating only women would lead to a decrease of 30% in HPV prevalence. Effectively implementing HPV vaccination in nation wide screening programs will lower prevalence of cervical cancer and its precursor lesions, but not completely eliminate it. After implementation it will take at least 20 years before this reduction in prevalence occurs. A lower prevalence of cervical cancer and its precursor lesions will then lead to an increase of false positive results. Detection of residual cervical cancers will therefore require screening tools with a high specificity. Morphology based Pap-smear analysis does not meet those criteria. It therefore seems to be of eminent importance that the search for, and development of new cervical cancer screening approaches are continued, now that cervical cancer prevalence is still high.

Addendum. Search strategy

We searched medline using the search terms “cervix neoplasms”, “base sequence”, “molecular sequence data”, “polymerase chain reaction”, “loss of heterozygosity”, “alleles”, “chromosomes, human”, “microsatellite repeats”, “oligonucleotide array sequence analysis” and “DNA, mitochondrial”. We limited ourselves to papers in English on human subjects, published until November 2005. After this date more recent papers that came to our attention were also incorporated in this review when relevance was apparent. Abstracts of the identified manuscripts were studied and of those manuscripts full text articles were selected that were considered relevant for this review. From the reference lists of the thus selected articles and by identifying related articles on Pubmed more relevant publications were selected. Preference was given to publications describing randomized clinical trials, meta-analyses or reviews or large prospective observational studies.

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