Innovative targets for diagnostics and therapy in cervical neoplasia

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SUMMARY

Cervical cancer is an important cause of death in women worldwide. Infection with high-risk human papillomavirus (HPV) has been identified as the main etiologic factor in cervical carcinogenesis.[1] An estimated number of 370,000 – 470,000 new cases occur per year, and 230,000 women die of cervical cancer per year[2-4] thus being the third to second most common cancer in women with large incidence differences between developed countries with cervical cancer screening programs and less developed countries without such programs. The cumulative lifetime risk for a woman to develop cervical cancer varies from 0.4% in Israel to 5.3% in Colombia.[5] Cervical cancer develops from cervical intraepithelial neoplasia (CIN). A CIN-lesion can either regress, persist or progress towards (micro)invasive carcinoma. Most of the low grade CIN lesions (CIN I) will regress, while in the long-term 12% to 40% of high grade CIN lesions (CIN II/III) will progress to squamous cell carcinoma.[6,7] Because no markers exist that identify those lesions that will progress, clinicians have felt compelled to treat at least all CIN II/III lesions.

Cytomorphologic examination of cervical smears is the most widely applied screening-method for cervical cancer and its precursors. Disadvantages are high numbers of false-negative and false-positive cervical smears. Parameters in cervical scrapings are needed which more accurately predict the presence of higher grade CIN or cervical cancer lesions to improve cervical cancer screening.

In **Chapter 2** available data on new approaches to cervical cancer screening such as quantitative cytochemistry, and detection of HPV DNA, loss of heterozygosity, microsatellite alterations, telomerase activity and DNA methylation were summarized. The potential of these approaches to replace or augment current Pap-smear screening was discussed. It was concluded that to date no other diagnostic tools are available that can cost-effectively replace or augment current Pap-smear screening. However, improvements may lay in the future. Implementation of liquid based cytology would aid the development of new technologies that can be performed on cervical scrapings. HPV DNA detection stands closest to implementation in nation-wide screening programs of all markers reviewed, but may also have important drawbacks. An interesting new technology that should be further developed is the detection of promoter hypermethylation of tumor suppressor genes by quantitative methylation specific PCR (see Chapter 7).

Telomerase is an enzyme that can form new short stretches of repeat nucleotides at the telomeric ends of chromosomes that were lost with each round of replication. Studies in tumour cell lines, as well as in human tumour specimens have shown that, in contrast to normal somatic cells, the vast majority of malignant cells (>90%) are characterized by increased telomerase activity.[8,9] Therefore determination of telomerase activity has been suggested for early cancer detection. In **Chapter 3** we studied whether detection of either telomerase and its components, or high risk human

papillomavirus (HPV) are of value in predicting the presence of CIN II/III in patients referred because of cervical cytology reports showing at most moderate dyskaryosis. The cervical smears of 50 patients with cytological borderline, mild or moderate dyskaryosis were analyzed. Telomerase activity was assessed by a commercially available TRAP-assay and its components human telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT) by reverse transcriptase-PCR. HPV was detected by GP5+/6+ PCR enzyme immunoassay. Histological findings on colposcopy guided biopsies or excised cervical tissue were regarded as pathological final diagnosis. Sensitivity and specificity for detecting CIN II/III were calculated. Twenty-eight patients (46%) were diagnosed with CIN II/III. In the patients diagnosed with CIN II/III, telomerase activity was detected in none, hTR in 88%, hTERT in 23% and high-risk HPV in 79%. As a diagnostic test none of the described analyses combined a sensitivity of at least 90% with a specificity of at least 90%. Despite the small numbers, calculation of the 95% confidence intervals excluded a combined sensitivity and specificity of at least 90% for any of the evaluated parameters. Therefore neither detection of telomerase or its components, nor detection of high risk HPV seem suitable for the triage of patients with borderline, mild and moderate cytological dyskaryosis.

In order to prevent increased morbidity associated with double modality treatment, early stage cervical cancer patients should be offered radical surgery only when there is a low likelihood for adjuvant radiotherapy. **Chapter 4** describes whether serum Squamous Cell Carcinoma-antigen (SCC-ag) analysis allows better preoperative identification of a group of patients with a low likelihood for adjuvant radiotherapy than currently used clinical parameters. In a historical cohort study, FIGO stage, tumor size and preoperative serum SCC-ag levels, as determined by enzyme immunoassay, were related to the frequency of postoperative indications for adjuvant radiotherapy in 337 surgically treated FIGO stage IB/IIA squamous cell cervical cancer patients. In patients with normal preoperative SCC-ag 16% of stage IB1 and 29% of stage IB2/IIA had postoperative indications for adjuvant radiotherapy, in contrast to 57% of stage IB1 and 74% of stage IB2/IIA patients with elevated (>1.9 ng/mL) serum SCC-ag ($p<0.001$). Serum SCC-ag was the only independent predictor for a postoperative indication for radiotherapy (odds ratio 7.1, 95% CI 4.2-12, $p<0.001$). Furthermore, in stage IB1 patients that did not have indications for adjuvant radiotherapy 15% of the patients with elevated preoperative serum SCC-ag levels recurred within 2 years in contrast to 1.8% of the patients with normal serum SCC-ag levels ($p=0.02$). It was concluded that determination of serum SCC-ag in early stage cervical cancer patients allows more refined preoperative estimation of the likelihood for adjuvant radiotherapy than current clinical parameters and simultaneously identifies patients at high-risk for recurrence when treated with surgery only. Therefore, preoperative serum SCC-ag analysis deserves implementation in clinical decision making.

Three different infectious agents of which a possible involvement in cervical carcinogenesis has been suggested in literature were studied. First, **Chapter 5** describes the prevalence of risk factors for and the impact on histologic changes of

bacterial vaginosis in patients with cytological abnormalities of the uterine cervix. Therefore 280 patients with dyskaryotic smears (mild, moderate or severe dyskaryosis) were asked to participate in the study, using a questionnaire to obtain data on smoking habits and sexual history. Bacterial vaginosis was diagnosed if the vaginal discharge produced a fishy odor upon alkalinization and if clue cells were seen in the wet smear. Furthermore, cervical scrapes were analyzed for the presence of HPV DNA, and cervical tissue specimens were analyzed for the presence and severity of (intraepithelial) neoplasia and the proliferation rate (mitotic index) of the lesion. *Chlamydia trachomatis* was identified by culture of an endocervical swab. Bacterial vaginosis was found in 56 (20%) of the patients. The presence of bacterial vaginosis was associated with the number of cigarettes smoked per day, age at first sexual intercourse, lifetime number of sexual partners, and current *Chlamydia trachomatis* infection. Number of cigarettes currently smoked per day and lifetime number of sexual partners were independent risk factors for the presence of bacterial vaginosis. There was no relation between the presence of bacterial vaginosis and HPV infection. Bacterial vaginosis did not influence the severity or the mitotic index of the neoplastic lesion found. In conclusion, in patients with dyskaryotic cervical smears, the prevalence of bacterial vaginosis is not increased, and bacterial vaginosis does not influence histologic changes. Bacterial vaginosis is therefore unlikely to be important in the etiology of cervical neoplasia, despite the similarity between its epidemiologic features and those of cervical HPV infection and cervical neoplasia.

Chlamydia trachomatis was the second infectious factor possibly involved in cervical carcinogenesis that was studied. In **Chapter 6** it was explored whether the presence of *Chlamydia trachomatis* antibodies was associated with the severity of neoplastic lesions in patients with cervical dyskaryosis. In a cross-sectional study serum samples were analyzed for antichlamydial antibodies by enzyme immunoassay. Cervical neoplasia was graded histologically. In 296 eligible patients referred because of cervical dyskaryosis between 1988-1993 no association was found between increasing grade of CIN and the presence of antichlamydial antibodies. The proportion of patients with antichlamydial antibodies was higher in patients with (micro)invasive carcinoma (13/14 (93%)) than in patients with CIN (101/282 (36%)). As the high prevalence of antichlamydial antibodies in patients with cervical carcinoma was not consistent with prevalences reported in recent literature, a second group of patients was analyzed (331 eligible patients in the period 1995-1999) in which the high prevalence of antichlamydial antibodies in patients with cervical carcinoma was not confirmed. These results suggest that the presence of circulating antichlamydial antibodies is not associated with the severity of neoplastic lesions and it seems unlikely that *Chlamydia trachomatis* has a role in the progression of cervical neoplasia.

The last and most important infectious parameter studied was HPV. HPV infection is the first identified essential step in cervical carcinogenesis, meaning that cervical cancer, with very rare exceptions, can not develop without HPV infection. Over 100 HPV types have been identified of which more than 35 types can be found in the genital tract and

of which 18 are associated with cervical carcinogenesis. Because of the important etiologic role of HPV numerous studies have been performed to identify factors involved in HPV infection and persistence. Different HPV detection techniques are available and might influence the results of epidemiologic studies.
A previous study comprising 304 patients referred because of abnormal pap smears showed that the prevalence of HPV rises with increasing histological severity of neoplasia, more cigarettes smoked per day and higher lifetime number of sexual partners in patients with cervical dyskaryosis using the GP5/6 primers based HPV PCR.[1] Data on the lifetime number of sexual partners and smoking behavior were obtained by questionnaire. HPV analysis was performed on cervical scrapes obtained at the enrolment visit. Some years after that initial epidemiologic study the highly sensitive SPF10 primers and Inno-LiPA (line probe assay) HPV prototype research assay became available for the detection and typing of HPV. In **Chapter 7**, using the SPF10-LiPA system, we re-analyzed the stored cervical scrapings of the earlier described study population. High-risk HPV was present in 288 (95%) patients. A total of 86 (30%) out of these 288 patients disclosed multiple types. HPV 16 occurred less often in multiple infections than was expected on the basis of chance alone. The grade of neoplasia was associated with the presence of oncogenic HPV, which depended on the presence of HPV type 16. No association was found between grade of neoplasia and the presence of multiple HPV types. Neither the lifetime number of sexual partners nor smoking were associated with high-risk HPV, the five most frequent HPV types separately or the presence of multiple types. We conclude that the association between the detection of HPV and the epidemiological risk factors, as found with the GP5/6 PCR in the past, could not be confirmed when using SPF10 PCR primers and LiPA HPV genotyping. We suggest that the number of sexual partners and smoking may be determinants of high HPV viral load rather than determinants of the presence of high-risk HPV per se.

It has become increasingly clear that silencing of tumor suppressor- or other cancer-associated genes by methylation of CpG islands is a common feature of human cancer including cervical cancer. In addition to the functional implications of gene inactivation in tumor development, these aberrant methylation patterns represent interesting targets for novel diagnostic approaches based on methylation sensitive PCR techniques which we tried to translate to clinical use in cervical cancer detection in **Chapter 8**. It was unknown whether a cervical scraping reflects the methylation status of the underlying epithelium. It was therefore unclear whether quantitative hypermethylation specific PCR (QMSP) on cervical scrapings could be used as a future screening method augmenting the current approach. The use of QMSP on cervical scrapings was explored by analyzing cervical scrapings and paired fresh frozen cervical tissue samples obtained from 53 cervical cancer patients and 45 controls. All scrapings were morphologically scored and analyzed with QMSP for the genes *APC, DAPK, MGMT* and *GSTP1*. *APC* mediates proliferative signals, *DAPK* is a proapoptotic gene and potentially inhibits metastasis, *MGMT* is a DNA repair gene and *GSTP1* is a detoxifying gene.[2] To adjust for DNA input, hypermethylation ratios were calculated against DNA levels of a reference gene.

Hypermethylation ratios of paired fresh frozen tissue samples and scrapings of cervical cancer patients and controls were strongly related. More cervical cancer patients than controls were *DAPK* positive ($p<0.001$). When 'screen positive' cutoff levels for ratios were defined to be above the highest ratio observed in controls, QMSP in cervical scrapings identified 32 of 48 (67%) cervical cancer patients. This feasibility study, in which cervical scrapings were analyzed for only four genes, demonstrates that QMSP holds promise as a new diagnostic tool for cervical cancer. The addition of more genes specifically methylated in cervical cancer are necessary to improve the sensitivity of the assay.

Increasing imbalance between proliferation and apoptosis is thought to be important in cervical carcinogenesis. The death ligands FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induce apoptosis by binding to their cognate cell-surface death receptors Fas or death receptor (DR) 4 and DR5. The aim of our study described in **Chapter 9** was to examine possible changes in expression of these death ligands and their receptors at different stages of cervical carcinogenesis. The immunohistochemical expression and localization of Fas/FasL and DR4/DR5/TRAIL were assessed in 11 normal, 15 cervical intraepithelial neoplasia (CIN) grade I, 15 CIN II, 13 CIN III and 25 (microinvasive) squamous cell cervical cancers. The number of apoptotic cells was determined by the use of morphological criteria and the number of proliferating cells by counting Ki-67 positive cells. A marked increase in proliferation as well as apoptosis percentage was found with increasing severity of neoplasia. In normal cervix and CIN I samples FasL, DR4, DR5 and TRAIL staining was mainly observed in the basal/parabasal layer, whereas Fas staining was localized in the superficial, more differentiated epithelial layer. Frequency of Fas positive staining decreased with increasing severity of CIN. In contrast, homogeneous FasL, DR4, DR5 and TRAIL expression throughout the lesions was more frequently observed in CIN III and cervical cancer. FasL, DR4, DR5 and TRAIL staining patterns were correlated, although TRAIL expression was more intense in low grade lesions. No association was found between death receptor or ligand expression with the percentage of apoptosis or proliferation. The loss of Fas and the deregulation of FasL, DR4, DR5 and TRAIL in the CIN-cervical cancer sequence suggest a possible functional role of these death ligands and receptors during cervical carcinogenesis. Their observed frequent expression present DR4 and DR5 as promising targets for innovative therapy modalities in cervical cancer.

FUTURE PERSPECTIVE

In the Netherlands a population-based cervical cancer screening program exists. Women aged 30-60 years are invited to be screened for cervical cancer once every five years. Cervical smears are taken by general practitioners. Patients are referred to a gynecologist for colposcopic examination if abnormalities are found during screening. Current morphology based cervical cancer screening is associated with significant false-positive and false-negative results. To date no other diagnostic tools are available of which is known that they can cost-effectively replace or augment current Pap-smear screening. In countries with low prevalence of cervical cancer, as in the Netherlands, it is hard to determine whether the decrease in cervical cancer deaths as a results of screening counterbalances the negative effects of screening. Cervical cancer screening will especially be of benefit in women in countries with high cervical cancer prevalences. Pap-smear screening seems the best screening tool currently available. HPV DNA detection stands closest to implementation in nation-wide screening programs of all currently known 'markers'. The main advantages of HPV screening would be the prolongation of the screening interval for HPV negative women and the possibility of triage for women with minor morphological abnormalities. In the next five years the results will become available of a Dutch radomized controlled trial that evaluates wether implementation of these two main advantages can improve the efficiency of cervical cancer screening.[16] However, low specificity for (progressive) high-grade CIN and cervical cancer as well as negative psychological effects of knowledge of HPV positivity are important drawbacks. Even if the trials that are underway will show cost-effectiveness of implementing HPV DNA testing in cervical cancer screening, new markers and technologies could theoretically still lead to major improvements.

If the high false-positive and false-negative numbers of cervical cancer screening would be reduced it will become more beneficial to screen women, even if they live in countries with a low cervical cancer prevalence. An interesting new cervical cancer detection approach is tumor suppressor gene hypermethylation analysis by QMSP. In this thesis we showed that it has theoretical advantages over current Pap-smear analysis and some other new screening tools based on molecular changes. Our first analysis in cervical scrapings was promising. Cervical scrapings reflect the hypermethylation status of underlying epithelium very well. Testing for only four genes, already 67% of cervical cancer patients were identified by QMSP. In prostate cancer *GSTP1* is methylated in more than 90% of the tumors and diagnostic tests are now being developed.[17] In light of these prostate cancer results we expect it to be possible that for cervical cancer a panel of genes can be identified, which will cover almost all cervical cancer cases. The available data and expectations should spur the search for more genes specifically methylated during cervical carcinogenesis. Once a (cervical) cancer sensitive and specific hypermethylation panel is composed it will be necessary to define 'screen positive' cutoff values for population-based screening purposes. Together with sensitivity and specificity of a screening test, the positive and negative predictive value of such a test in a population is determined by the prevalence of disease in that

population. It may therefore be a rational approach to let the QMSP cutoff values depend on the prevalence of cervical cancer in a country. Further studies should clarify wether QMSP can also be used to identify progressive CIN II/III lesions.

The prevalence of cervical cancer may show a drastic change in the future, because primary prevention of cervical cancer by effective prophylactic HPV vaccines may become possible.[18] When it would be implemented, HPV vaccination is supposed to lower the prevalence of cervical cancer and its precursor lesions dramatically. If sensitivity and specificity of our screening tools will not be changed in that situation, positive screen results will thereby be more likely to represent false positives. However, one may still want to screen for residual CIN/cervical cancer patients in the hypothetical scenario that HPV vaccination is implemented. Reasons for ongoing screening may be that immunization will fail in some women, women may refuse to be vaccinated and probably only vaccines against HPV 16 and 18 will be developed. It is hard to estimate the prevalence of cervical cancer once HPV 16 and 18 vaccination is implemented in our health care system. Even when this implementation lays in the near future it will however take at least decades before it will result in a decrease of cervical cancer cases.

In this thesis we show that it is possible to refine existing treatment protocols for cervical cancer by serum detection of SCC-ag. The pretreament level of serum SCC-ag in cervical cancer patients was already known to be of prognostic value. Determination of serum SCC-ag levels in patients with early stage cervical cancer also allows more refined preoperative estimation of the likelihood for adjuvant radiotherapy than current clinical parameters and simultaneously identifies patients at high-risk for recurrence when treated with surgery only. In our opinion preoperative serum SCC-ag analysis should now be implemented in clinical decision making.

The etiology of cervical neoplasia starts to be unraveled and new technologies may accelerate our understanding of cervical carcinogenesis. It will be an ongoing challenge to find ways by which these improvements will help us to refine screening and treatment policies for individual patients.

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