PhD Thesis

COMBINATION OF CYTOLOGICAL AND MOLECULAR METHODS FOR IMPROVEMENTS IN CERVICAL CANCER PREVENTION

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INTRODUCTION

The main purpose of cytological screening by Papanicolau (Pap) smears is to identify women with cervical lesions that confer an increased risk to cervical cancer and treat them adequately. Consensus exists that women with high-grade cytological lesions should be referred immediately for further exploration. There is sufficient evidence that screening for cervical cancer using the Pap-test does prevent death and reduces incidence of this disease. However, current data indicate that the plateau of what can be achieved by conventional cytology is now being reached and further reductions in disease incidence and mortality rates require new technologies or more efficient screening strategies.

The recognition that the vast majority of cervical cancer cases worldwide are caused by persistent infections with certain oncogenic types of human papillomaviruses (HPV) has led to the development of diagnostic applications for HPV testing as an adjunct to cytology aiming at i) increasing the sensitivity of cytology screening, ii) triaging cases with equivocal cytological smears and iii) surveying women after surgical treatment of the disease. However, the management of women with minor cytological abnormalities remain controversial and results concerning the utility of HPV triage for women with equivocal cytology are inconsistent.

There is an increasingly large research literature on possible applications and combinations of new visual, microscopical, and virological screening methods for the prevention of cervical cancer. In addition to cytology, a second sensitive test can be used sequentially as a triage method to reduce the number of false screen-positives requiring referral as well as to restrict false cytology negatives with prevalent cervical neoplasias.

However, even the highly sensitive test combination of cytology and HPV genotyping cannot assess the biological potential of prevalent cervical intraepithelial neoplasia. An ideal test combination would indicate that an oncogenic HPV virus has already enhanced genetic

instability and rendered cells susceptible to malignant transformation and consequent progression if left untreated.

Consequently, there is a need for alternative protocols including enhanced cytology, human papillomavirus (HPV) tests or surrogate biomarkers of neoplastic transformation as well as test-combinations to enhance screening accuracy.

To address these issues, we have developed a risk-adapted multimodal cervical screening and management protocol at the Institute of Pathology in Bonn-Duisdorf including a combination of liquid-based cytology, PCR-based dynamic HPV genotyping and DNA cytometry and introduced it in the routine clinical practice for the first time in Germany.

By using this protocol, the main goals of the present project were threefold: (1) to increase our basic knowledge on morphological and molecular steps of cervical carcinogenesis, (2) to enhance the effectivity of cervical screening and (2) to define prognostic markers for early CIN (cervical intraepithelial neoplasia) behaviour and for post-treatment surveillance.

AIMS

I. To characterise cytomorphological and molecular basic features of HPV infection by defining associations between non-classical HPV-related cytomorphological signs and

   i) HPV genotypes

   ii) immunohistochemical expression of HPV L1 capsid protein

   iii) detection of HPV DNA using in situ hybridisation

   iv) presence of HPV E6/E7-mRNA transcripts

II. To determine HPV genotype distribution and

   i) the spectrum of cervical lesions induced by low-risk and undefined-risk HPVs

   ii) the prevalence of rare oncogenic HPV genotypes in cervical pre-cancer and cancer

   iii) the prevalence of HPV types with undefined oncogenic potential in cervical pre-cancer and cancer

III. To evaluate the efficiency of a risk-adapted multimodal screening and management strategy (the Bonn-protocol) for cervical cancer prevention in a routine screening population of about 30.000 women

MATERIALS AND METHODS

Patients and samples

1) office-based routine screening population of 31031 women (Bonn-region, West-Germany)
2) triage population of 5943 women, who received PCR-based HPV-testing
3) case series of different size with different grades of cervical pathologies for methodical studies

Procedures

1) Cytology

Thin-layer preparations for liquid-based cytology were made using the ThinPrep technique. Cytological diagnoses were classified according to the modified Munich II Cytological classification (standard in Germany) and converted into the Bethesda 2001 terminology.

2) Histology

Gross and histological processing of surgical specimens were performed according to standardised surgical pathology protocols, histological diagnoses were made using the WHO classification.

3) PCR-based HPV DNA detection and genotyping by sequencing

HPV DNA detection was directly performed from residual liquid-based cytology material by PCR-based assays using the MY09/MY11 consensus primers and the GP5+/6+ general primer system in combination with automated PCR fragment analysis. Results were compared with documented virus sequences available in GenBank database using the BLAST program. The assignment of oncogenic potential of HPV types was based on the epidemiological risk

assessment of HPVs in the development of cervical cancer according to established consensus from the literature.

4) DNA image cytometry

DNA ploidy measurements were carried out from the same liquid-based cytological material that was used for cytology and HPV-testing after re-staining the slides according to the Feulgen-method. The DNA content of at least 100 dysplastic epithelial cells and 30 normal-appearing intermediate cells for reference was measured interactively on individually focused nuclei using the ACAS cytometer system (Ahrens, Bargtheide, Germany).

5) In situ hybridisation

- To characterise cytomorphological and molecular basic features of HPV infection, the INFORM HPV III Family 16 Probe (B) was used in the BenchMark Staining Platform (Ventana Medical Systems) according to recommendations of the manufacturer. The probe cocktail has demonstrated affinity to the following HPV genotypes: 16, 18, 31, 33, 35, 39, 51, 52, 56, 58 and 66.
- To characterise chromosomal imbalance in HPV infected cells showing polyploidisation, the fluorescence in situ hybridisation (FISH) technique was used with pericentromeric probes specific for chromosome 3 and 17.

6) Immunohistochemical detection of HPV L1 capsid protein

The reactions were performed using the Cytoactive® HPV Screening Set (Cytoimmun, Germany) as recommended by the manufacturer.

7) HPV mRNA detection

Oncogene E6/E7 mRNA from HPV types 16, 18, 31, 33 and 45 was identified using the commercially available PreTect HPV-Proofer kit (NorChip AS Norway) based on real-time NASBA technology according to recommendations of the manufacturer.

RESULTS

Until submission of the dissertation we obtained the following results to the main issues investigated:

I. Cytomorphological and molecular basic features of HPV infection

1. Evaluation of "non-classic" cytomorphological signs suggestive of HPV effect in minimally abnormal cervical smears improves the sensitivity of cytology for detecting HPV infection at primary screening. In particular, mild nuclear changes had 100% sensitivity and 100% negative predictive value for PCR-detected HPV infection, using the MY09/MY11 consensus primers and the GP5+/6+ general primer system. Based on cytomorphology alone, no difference could be made between type specific HPV infections in cases with minimal cytological abnormalities that further underline the importance of genotypic analysis of HPV positive cases.
2. The INFORM HPV III Family 16 probe (B) contains a cocktail of HPV genomic probes in a formamid-based diluent. The intended targets are the common high-risk HPV genotypes found to be associated with cervical neoplasia. The probe cocktail has demonstrated affinity to the following genotypes: 16,18,31,33,35,39,45,51,52,56,58 and 66. The presence of HPV-DNA is demonstrated when either the episomal or integrated pattern is found within the nuclei of cervical epithelial cells. This method is very sensitive and shows positivity already at few viral DNA copy number. We could detect HPV-DNA both in cases with latent HPV infection showing non-classic cytological alterations and dysplastic epithelium.

3. HPV-induced genetic instability results in polyploidisation as well as in low frequency random chromosome aberrations in squamous cells. We analyzed whether highly polyploid/aneuploid cells reflect genomic changes at the chromosomal level. Thirteen samples with a cytological diagnosis of HSIL were analyzed for HPV type and DNA content. Hyperdiploid cells with >5c and >9c DNA content were further analysed for numerical aberrations of the chromosomes 3 and 17 by fluorescence in situ hybridisation (FISH). The FISH analysis demonstrated frequent polysomies. The rate of aneusomy was significantly higher in cells with >9c than in cells with >5c DNA content or in normal diploid cells. The imbalance of chromosome 3 and 17 copy number was also increased in cells with >9c DNA content. Moreover, in 3/13 HSIL samples analysed, recurrent abnormal chromosome 3/17 ratio was demonstrated in a significant part of the cells, indicating their common origin. These chromosomal aberrations may represent early changes in cells with tumorigenic potential.

4. HPV L1 capsid protein detection by immunochemistry was used to distinguish between productive and non-productive HPV infection according to the grade of the lesions. Routinely stained cervical smears (liquid based) of 204 women showing mild to severe dysplastic lesions (LSIL/HSIL) were immunochemically stained with a panreactive HPV-L1-specific monoclonal antibody (Cytoactiv®). HPV genotyping was preformed with the PCR method as described previously. In 99 cases, we could detect a positive immunostaining indicating productive infection. Sixty-nine of the cases (69.70%) were LSIL, and 30 cases (31.30%) were HSIL. Sixty-three of the cases (63.64%) were di-/polyploid and 36 (36.36%) aneuploid. According to the literature, non-productive high-grade aneuploid lesions have a high progressive potential and the Cytoactive test can be used in combination with cytology, HPV genotyping and DNA cytometry for separating patiens with progressive lesions from those with lesions which are more likely to regress for the follow up and the clinical management.

5. In HPV mRNA expression studies, there were highly significant associations between HPV genotypes and the mRNA type expressed in the samples. The diagnostic validity of the HPV mRNA (NorChip) test for detecting HPV infection was as follows: sensitivity 61.3 %, specificity 100%, positive predictive value (PPV) 100% and negative predictive value (NPV) 14.3%. The prognostic power of the NorChip Test for predicting cytological disease progression was: sensitivity 82.35 %, specificity 68.75%, positive predictive value (PPV) 73.68 and negative predictive value (NPV) 78.57%. The detection of HPV E6/E7 mRNA indicates HPV oncogenic activity and is therefore a more relevant clinical indicator of the development of cervical cancer than the detection of the presence of HPV DNA alone. Thus, HPV E6/E7 mRNA expression may be used as a clinically predictive marker to identify women at risk of developing high-grade cervical dysplastic lesions and cervical carcinoma

II. HPV genotype distribution and the spectrum of HPV-induced cervical lesions

1. Upon investigating the spectrum of cervical diseases induced by low-risk (LR) and undefined-risk (UR) HPVs, the most frequent cytological diagnosis was ASC-US (63%) followed by LSIL (23%), negative cases for intraepithelial lesion or malignancy (no-ASC) (9%) and HSIL (5%). No carcinoma was detected. The distribution of LR- an UR-HPV infections was not significantly different in the various cytopathological groups. All negative smears (no-ASC) and ASC-US cases showed at least one but generally more minor non-classical HPV-induced cytological alterations. The majority of LSIL and HSIL cases showed a combination of both classic (koilocytosis and dyskeratosis) and non-classic HPV-signs.
2. There was no “negative cytology” in HPV positive cases! Even in the absence of cellular atypia, minor non-classic signs of HPV-effect could be detected in the smear, if carefully assessed.

3. Overall, 30 different HPV types could be detected in the strictly selected group of patients harbouring mono-infections with LR- or UR-HPVs only. Three of the 19 UR-HPVs were defined as novel HPV genotypes.
4. After re-classifying our data according to the phylogenetical grouping of known HPVs, 12 of 19 UR-HPVs could be assigned either as LR types (HPV 32, 62, 83, 84, 86, 87, 91, 74) or probable high-risk types (HPV 69, 30, 67, 34). Infection with probable HR-HPVs signalise an increased risk of progression to HSIL and cancer. This highlights again the importance of HPV genotyping.

III. Efficiency of a risk-adapted multimodal screening and management strategy (the Bonn-protocol) for cervical cancer prevention

1. Our preliminary results from the year 2002 showed that the combination of cytology, HPV genotyping and DNA cytometry resulted in an increase of positive predictive value (PPV) up to 88.2% for moderate to high-grade cervical dysplasias and carcinomas (≥CIN2) compared to single tests or double combinations.
2. This combined approach had the additional benefit of being able to predict the possible outcome of histologically proven CIN1 lesions detected as false positives by single tests. The positivity for HR-HPV and DNA aneuploidy in a CIN1 lesion signalizes a high risk for progression, whereas HR-HPV positivity with diploid DNA content indicates a probable benign course.
3. Our multimodal cervical screening protocol permits identification of those women with low-grade squamous intraepithelial lesions (LSIL/CIN1) likely to progress at earlier and curable stage of disease and distinguish them from transient minor lesions caused by productive HPV infection. Thus, many women could be prevented from unnecessary colposcopy and conisation while others being at high risk for potential progressive diseases could be treated timely.

LIST OF PUBLICATIONS

Original articles [impact factor: IF (2005)]:

1. Poser I, Dominguez D, de Herreros AG, Varnai A, Buettner R, Bosse AK. Loss of E-cadherin expression in melanoma cells involves upregulation of the transcriptional repressor Snail. J. Biol. Chem. 2001; 276: 24661-24666.

   IF: 6.854

2. Varnai A, Bollmann M, Griefingholt H, Bollmann R, Schmitt C, Speich N, Decker D: HPV in anal squamous cell carcinoma and anal intraepithelial neoplasia (AIN): Impact of HPV analysis of anal lesions on diagnosis and prognosis. Int J Colorectal Dis. 2006; 21:135-42.

   IF: 1.749

3. Bollmann M, Varnai A, Griefingholt H, Bankfalvi A, Callenberg H, Speich N, Schmitt C, Bollmann R: Predicting treatment outcome in cervical diseases using liquid-based cytology, dynamic HPV genotyping and DNA cytometry. Anticancer Res. 2006; 26:1439-46.

   IF: 1.604

4. Várnai AD, Bollmann M, Bánkfalvi A, Griefingholt H, Pfening N, Schmitt C, Pajor L, Bollmann R: The Spectrum of Cervical Diseases Induced by Low-risk and Undefined-risk HPVs: Implications for Patient Management. Anticancer Res 2007; 27: 563-570.

   IF: 1.604

5. Walgenbach-Bruenagel G, Tolba RH, Varnai AD, Bollmann M, Hirner A, Walgenbach KJ: Detection of lymphatic invasion in early stage primary

   Colorectal cancer with the monoclonal antibody D2-40. Eur Surg Res 2006; 38: 438-444

   IF: 0, 755

6. Mehes G, Kovacs G, Kajtar B, Lacza A, Varnai A, Losonczy H, Pajor L: Karyotype complexity and VH gene status in B-cell chronic lymphocytic leukemia. Haematologica. 2006; 91(10):1430-1.

   IF: 4,192

Review

1. Bollmann R, Bankfalvi A, Varnai AD, Bollmann M: Risiko-adaptierte multimodale gynäkozytologische Krebsvorsorge – Der Pap-Test der Zukunft. Pathologe, 2007 (in press)

Book chapter

1. Bollmann R, Varnai AD, Bankfalvi A, Bollmann M: Alternative approaches to cervical cancer prevention: risk-adapted multimodal laboratory cervical screening. In: New Research on Cervical Cancer, Eds: George Z. Rolland, Nova Science Publishers, Inc., 2007.

Congress Posters & Presentations:

1. **Varnai A**, Bosserhoff A, Büttner R.: Molecular Alterations of the E-Cadherin Gene in Melanoma and Colorectal Carcinoma Cell Lines. Arch. Hung. Med. Assoc. America 10 (2): 12-13, 2002.
2. Bollmann R, Méhes G, **Varnai A**, Bollmann D, Speich N , Bollmann M: DNA aneuploidy/polyploidy is associated with oncogenic HPV infection in ASCUS and SIL. Pathol. Res. Pract. 200 (4): 356, 2004.

3. Méhes G, Speich N, **Varnai A**, Bollmann D, Bollmann M, Bollmann R: Chromosomal aberrations accumulate in polyploid cells of high-grade squamous intraepithelial lesions (HSIL). Pathol. Res. Pract. 200 (4): 356, 2004.
4. Speich N, Schmitt CH, **Varnai A**, Bollmann D, Bollmann R, Bollmann M: Human papillomavirus (HPV) study of 2.916 cytological samples by PCR and DNA sequencing: Genotype spectrum of patients from the West-German area. Pathol. Res. Pract. 200 (4): 357, 2004.
5. **Varnai A**, Bollmann D, Bollmann O, Bollmann R: Dynamic Telemacropathology with internet cameras. 7th Telepathology Congress, Poznan, 9th July 2004.
6. **Varnai A**., Speich N., Bollmann R., Bolmann M.: Das HPV-genotyp-Spektrum in Nordrhein-Westfalen: Ergebnisse der HPV-PCR und nachfolgender DNA-Sequenzierung an 2916 zytologischen Präparaten. 55 Kongress der Deutsche Gesellschaft für Gynäkologie und Geburtshilfe, Hamburg, 14.-17.September 2004
7. **Varnai A**, Speich N, Bollmann R, Bollmann M, Méhes G: Chromosomal Aberrations Accumulate in Polyploid Cells of High Grade Squamous Intraepithelial Lesions. Hungarian Med. Assoc. America, 36th Annual Scientific Meeting. Sarasota, Florida, October 24-29, 2004.
8. **Varnai A**, Griefingholt H, Bollmann R, Speich N, Bollmann M: HPV in Anal Squamous Cell Carcinoma and in Anal Intraepithelial Neoplasia (AIN). Hungarian Med. Assoc. America, 36th Annual Scientific Meeting. Sarasota, Florida, October 24-29, 2004.
9. **Varnai A**, Bollmann M, Griefingholt H, Bollmann R, Schmitt C, Speich N, Türler A, Decker D: Diagnostische und prognostische Bedeutung der HPV- Analyse analer Läsionen. 60. Jahrestagung der DGVS (Deutsche Gesellschaft für Verdauung und Stoffwechselerkrankungen) Köln, September 2005.
10. **Varnai A**D, Bollmann M, Bollmann B, Trosic A, Speich N, Schmitt C, Bankfalvi A, Bollmann R. Validity of non-classic cytomorphological signs for the prediction of HPV

infection in minimally abnormal cervical smears. 90. Tagung der Deutschen Gesellschaft für Pathologie, Berlin, April 19-21, 2006

11. **Varnai AD**, Bollmann D, Bollmann M, Griefingholt H, Bankfalvi A, Bollmann R. A combination of enhanced cytology, HPV genotyping and DNA cytometry enables individual prediction of clinical outcome of residual cervical diseases. Magyar Orvosok és Gyógyszerészek Világtalálkozója, Debrecen, June 8-10. 2006.