MULTIPLE HUMAN PAPILLOMAVIRUS INFECTIONS IN ROUTINE CYTOLOGY-BASED CERVICAL SCREENING AND HISTOLOGY PRACTICE: EPIDEMIOLOGY AND HISTOMORPHOLOGICAL INVESTIGATIONS

Ph.D. Thesis

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ABBREVATIONS

AEC ..... amino-ethyl-carbasol
AP ..... alkaline phosphatase
ASCUS ..... Atypical Squamous Cells of Undetermined Significance
BCIP ..... bromo-chloro-indoyl-phosphate
BLAST ..... Basic Local Alignment Search Tool
bp ..... base pair
CI ..... confidence interval
CIN ..... cervical intraepithelial neoplasia
CISH ..... chromogenic in situ hybridization
DIG ..... digoxigenin
DNA ..... deoxyribonucleic acid
E region/gene ..... early region /gene
FAM 6-Carboxyfluorescein
H&E ..... hematoxilin and eosin
HPV ..... Human papillomavirus
HR- ..... high-risk-
HRP ..... horseradish peroxidase
HSIL ..... high grade squamous intraepithelial lesion
IHC ..... immunohistochemistry
INNO-LiPA ..... Innogenetics Line Probe Assay
ISH ..... in situ hybridization
L region/gene ..... late region/gene
LA ..... Linear Array
LBC ..... liquid based cytology
LR- ..... low-risk -
LSIL ..... low grade squamous intraepithelial lesion
NBT ..... nitroblue tetrasolium
OR ..... Odds ratio
PCR ..... Polymerase Chain Reaction
UR- ..... unknown-risk -
VAIN ..... vaginal intraepithelial neoplasia
WHO ..... World Health Organisation

INTRODUCTION

In our present knowledge the vast majority of cervical carcinoma and its precursors worldwide are causally associated with persistent infections by high-risk human papillomaviruses (HR-HPV). Recent studies of the biological behaviour of viri and the natural history of HPV infections have reflected on the importance of genotyping, especially regarding the fact, that a significant proportion of infections are caused by different genotypes transiently and consecutively.

20–40% of HPV-positive women are reported to be infected with multiple HPV types. Epidemiologic studies of multiple infections, in the light of recent findings on possible synergism between HPV types in the induction and progression of cervical lesions and in an era where HPV vaccination commences have significance. Longitudinal studies have suggested that the one time, cross-sectional detection of HPV types may underestimate the cumulative diversity of exposure to HPV in the long term.

At the moment there is no "gold standard" for HPV detection and genotyping. Despite its recognised high analytical sensitivity, the worldwide used consensus PCR-based protocols may have missed cases harbouring a virus load lower than the threshold of detection. There is also a significant proportion of women who had positive results by consensus - PCR but their sample was not tested by genotyping, due to many reasons.

The apparent disparity in HPV prevalence in the literature highlights the fact that rates of HPV infection in a specific setting vary considerably according to many factors, such as the characteristic of investigated population and the HPV detection methods.

In routine diagnostic practice, several possible applications and combinations of new visual, microscopical, and virological screening methods have already been applied for the prevention of cervical cancer. However, even colposcopy and the highly sensitive ancillary tests of combined cytology and HPV genotyping cannot define the specific areas of the cervix where the specific HPV - type is contained, and where the least regressive CIN3/CIS is located, especially in the cases of multiple HPV infections. Only the histological examination may be able to confirm the distributional localisation of different genotypes and the grade of different HPV - induced squamous lesions.

AIMS

Based on the statistical analysis of data obtained from patients followed in the risk-adapted screening program of Germany's Bonn region and the re-evaluation of their histological samples, we sought answers to the following questions:

I. Epidemiologic characteristics of multiple HPV infections

a) What is the frequency of multiple HPV infections within the examined population and what is the distribution of genotypes?
b) Are there differences between methods used for virus detection regarding the number of different, identifiable HPV genotypes?
c) Are there differences between different age-groups regarding the incidence and prevalence?

II. Characteristics of persistent HPV infections

a) What are the cytologic and histologic characteristics of persistent infections lasting significantly longer than the elimination period, that are caused by a single genotype?
b) Are the differences between genotypes regarding the duration of the infection?
c) What are the differences between infections caused by a single or multiple HPV genotypes?

III. Morphological spectrum of lesions of the uterine cervix infected by multiple HPV genotypes – viral detection

a) What kind of histological lesions can be identified in cases of multiple HPV infection?
b) Can the presence of HPV be demonstrated at the histological level?
c) Can the different genotypes be localised histologically? What is the topographical distribution of the different genotypes?

MATERIAL AND METHODS

Patients and samples

Cytological and histological samples of patients selected from the population of 7.5 years of routine gynecological oncocytological screening based on elevated risk for cervical cancer were used for the purposes of the study. 11971 liquid-based cytological samples of 8090 patients and the HPV DNA tests performed from the samples were analysed.

I. phase: 489 multiple HPV infected women, 592 HPV DNA test, 589 cytology; histological examination in127 patients

II. phase: 6017 LBC sample and HPV DNA test of 2136 women: 100 women / 415 HPV DNA test; histology in 46 patients

III. phase: 97 histological preparations of 127 women; 12 patients /histology (HPV16+31)

Diagnostic procedures and methods

1) Cytology: For liquid-based cytology (thin-layer) preparations were made using the ThinPrep 2000 processor. Cytological diagnoses were classified according to the modified Munich II Cytological classification and converted into the Bethesda 2001 terminology.

2) HPV DNA detection was directly performed from residual liquid-based cytology material by 5 different PCR-based assays:

a) PCR-based HPV DNA detection and genotyping by sequencing: 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.

b) HPV type-specific PCR: Type specific PCR was performed for the common HPV types 6, 11, 16, 18, 31, 33, and 51 according to published protocols [van den Brule et al., 2002] with modifications [Speich et al., 2004]. All forward primers used in PCRs were labeled with FAM; this allowed analysis of PCR fragments by fluorescence capillary electrophoresis.

c) Roche Linear Array HPV genotyping test: involves PCR amplification of target DNA, followed by nucleic acid hybridization using a reverse line blot system for the simultaneous detection of 37 anogenital HPV genotypes. The LA HPV genotyping test amplifies a region of approximately 450 bp in length within the L1 gene of the HPV genome.

d) INNO-LiPA HPV genotyping v2 test: is able to detect 24 HPV types by hybridization of an amplified fragment of the HPV L1-region to immobilized oligonucleotides on a membrane strip.

e) PapilloCheck test: is based on the detection of an amplified fragment of the E1 gene of HPV. For analysis of the data the CheckReport analysis-software was used. The PapilloCheck Test detects 24 HPV types.

3) Histology

Gross and histological processing of surgical specimens were performed according to standardised surgical pathology protocols, histological diagnoses were made using the WHO (1975) and CIN (Richart) classification.

a) H&E staining: conventional

b) Immunohistochemical detections (IHC):

- p16 ^{INK4a} : Detection of human p16 ^{INK4a} protein was performed using CINtec p16^{INK4a} antibody (*mtm Laboratories, Germany)*.
- Ki67: *(Mib1)* Demonstration of HPV-induced cell proliferation *(NeoMarkers, Fremont, USA)*.
- Detection of HPV L1 capsid protein: The reactions were performed using the Cytoactive® HPV Screening Set (Cytoimmun, Germany) as recommended by the manufacturer.

All tests were done using standard, streptavidin-biotin-peroxidase based indirect technique as recommended by the manufacturer.

c) In situ hybridisation (ISH): To characterise the histo(cyto)morphological and molecular basic features of HPV infection, the following techniques were used:

- INFORM HPV III Family 16 Probe (B) ISH: 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.
- manually: a) HPV16 biotin, b) HPV31 digoxigenin labelled probes were used with a) avidin-HRP + AEC, b) anti-dig-AP + NBT/BCIP enzyme-substrate reactions.

RESULTS AND CONCLUSIONS

I. Epidemiologic characteristics of multiple HPV infections

In the present study, the prevalence and type-specific composition of multiple HPV infections were investigated in a risk adapted, multimodal protocol based routine cervical screening population from the federal state North Rhine-Westphalia in West Germany both at a cross-sectional level and longitudinally.

Four hundred eighty-nine out of 8090 women were diagnosed with multiple HPV infections once or repeatedly. During the 7.5-year study period, the cumulative prevalence of HPV co-infections was 15.3% in contrast to the cross-sectional prevalence of 3.8% at single visits. The overall cumulative prevalence within the cohort of all women screened was 6.9%. Using consensus PCR with sequencing and type-specific PCRs, two to three HPV types were detected simultaneously, whereas broad spectrum methods detected up to seven different genotypes in one sample. Nevertheless, the most common pattern of co-infection occurred with two to three HPV types irrespective of the age of the patient, cytology and histology of the lesions and the method used. Altogether, 45 different HPV genotypes were detected using different HPV detection methods. The most common genotypes detected were HPV16, 31, 53, 51, 52, and 66, and the most common pattern of co-infection was double infection with HPV16 and 31.

The highest prevalence of multiple HPV infection occurred in women between 25 and 34 years of age (35.5%). There was no significantly increased risk for multiple HPV infection in younger women (<30 years) over the older ones.

The prevalence of multiple HPV infection was significantly lower in HSIL (14.7%) than in LSIL (45.7) and ASCUS (39.6%) respectively.

Histologically, the highest prevalence of multiple HPV infection was detected in high grade cervical intraepithelial neoplasias (CIN3; 43.3%).

Our results show that rates and patterns of multiple HPV infections are largely dependent on the methodology used and the time interval between tests.

II. Study of longer than 18 months type-specific human papillomavirus persistence

In this phase of the study from a screening population of 8090 women, a strictly selected cohort of 100 patients with ≥18-month persistent, type-specific HPV infection were prospectively followed-up for a mean of 35.52 months (± 13.0).

Altogether, 21 different genotypes were detected. Seventy-two percent of women were infected with high-risk (HR)-HPVs, 24% with low-risk (LR)- and 4% with unknown risk (UR)-HPV types. The mean duration of infections showed considerable variation among the different HPV types and risk groups detected and ranged between 19.7 and 54.3 months.

44% of cases had co-infections with multiple HPV types. 95% of multiple infections harboured at least one HR-HPV. In the majority of multiple infections (75%) the persistent type was HR-HPV type, of them, the most common types were HPV16 (18.2%).

The prevalence of HR-HPV infection was directly correlated with increasing grade of cytological atypia, i.e., 66.6% were detected in ASCUS, 78.6% in LSIL and 88.8% in HSIL. Cytological progression into LSIL and HSIL occurred in 33% of women. Eleven cases that progressed into HSIL during follow-up.

The risk of progression in LSIL and HSIL was the highest for infections with HR-HPVs, whereas cases with LR- and UR-HPVs tended to regress or remained unchanged during follow up. There was no significant difference between risk of progression into HSIL either among women younger or older than 30 years nor in mono- and multiple infections.

In histologically proven cases 60% had ≥CIN2/VAIN2 lesions, 36% of them were associated with multiple types. 50% of patients with multiple types had ≥CIN2 lesions and harboured persistent HPV16. In monoinfections 55% of histologically proven ≥CIN2/VAIN2 cases harboured persistent HPV16.

Summary: Detecting long-term persistent HPV infections by genotyping may help identify women with cervical intraepithelial lesions who are at lower and higher risk of developing high grade pre-cancer and cancer.

III. Morphological spectrum of lesions of the uterine cervix infected by multiple HPV genotypes – viral detection

Upon investigating the spectrum of cervical diseases induced by multiple HPV genotypes the following lesions were identified: invasive squamous cell cancer, cervical intraepithelial neoplasia (CIN1-3) and adenocarcinoma in situ (in two cases).

In each sample, either classic (koilocytosis, dyskeratocytosis) or a combination of non-classic (nuclear abnormalities, cytoplasmic abnormalities and disorders of keratinisation, suprabasal mitotic figures) histo(cito)morphological HPV signs were detected in a carcinoma or dysplastic free, normal appearing or hyperplastic ecto/endocervical epithelium. Based on morphology alone, no difference could be made between type specific HPV infections.

Immunohistochemical ($p16^{INK4a}$, Mib-1, HPV L1-capsid) and in situ hybridization methods were used to visualize the HPV-induced genetic instability resulting in cell-cycle disorders and the presence of common high-risk HPV genotypes in dysplastic, minimally abnormal and premalignant hyperplastic lesions of the uterine cervix. Biotinylated and digoxigenin labelled type specific HPV DNS probes against HPV16 and HPV31 were used for detection tissue localisation of different genotypes.

The variable histomorphological characteristics of HPV infection and the topographical distribution of the viri have been compiled in histological graphs (Figure 1).

Our morphological observations regarding alterations present in every HPV-infected tissue may help the routine diagnostic work by highlighting the etiological role of HPV.

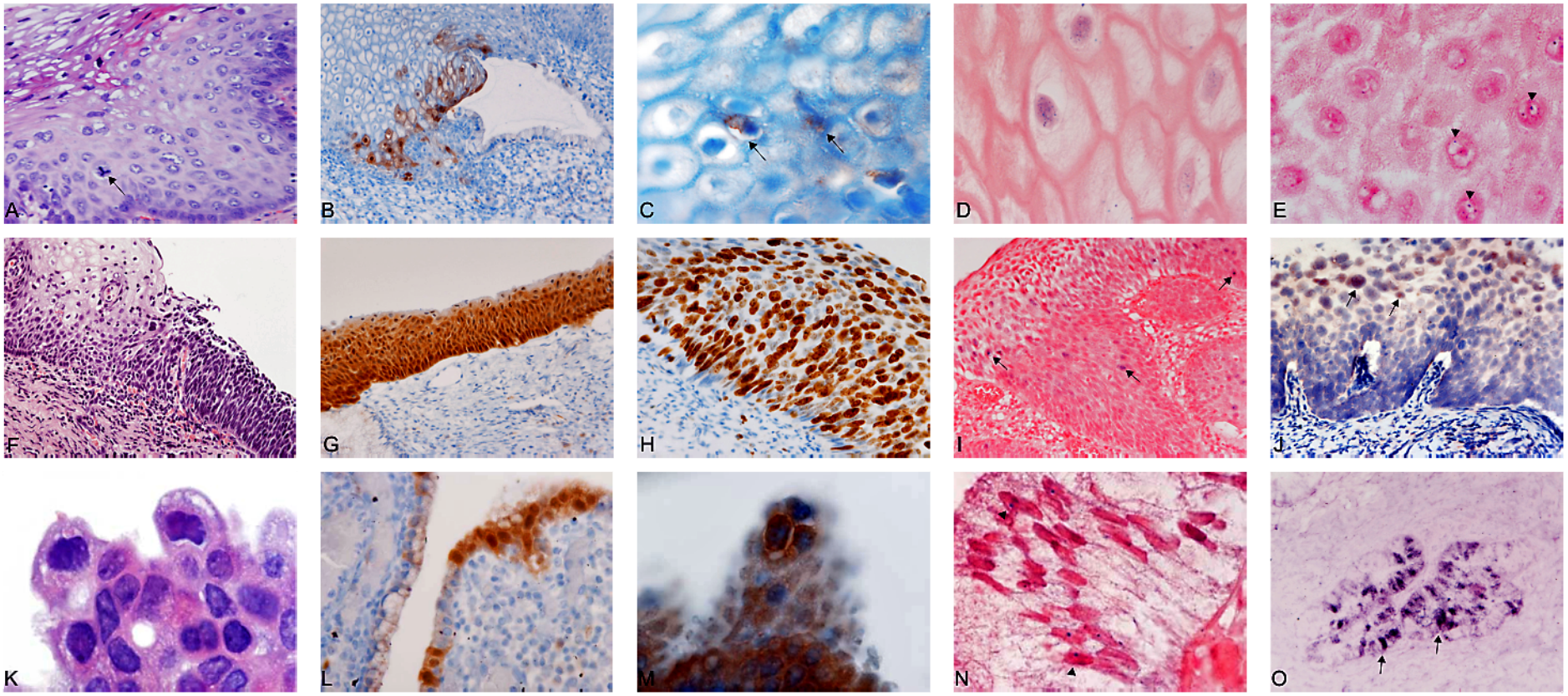

Figure 1. HPV induced histomorphological alterations and cell-cycle disorders of the cervix

A) Non-classic cytological signs in HPV induced hyperplastic cervical epithelium [note parakeratosis and atypical mitosis in the suprabasal layer] [arrow] (H&E stain,200x); **B)** Focal $p16^{INK4a}$ positivity in hyperplastic squamous epithelium (IHC,100x); **C)** HPV L1 capsid protein positivity [arrows] in hyperplastic squamous cells (Viroaktiv® IHC,400x); **D)** HR-HPV DNA detection in abortive koilocytes (CISH,1000x); **E)** Low copy infection with HR-HPV in 'normal-appearing' squamous cells [arrows] (CISH,1000x); **F)** Distinct border between hyperplastic and HPV induced high-grade (CIN3) dysplastic epithelium (H&E stain,200x); **G)** Diffuse $p16^{INK4a}$ staining in CIN3 lesion (IHC,200x); **H)** High proliferative activity in CIN3 lesion (Ki67IHC,200x); **I)** Diffuse (episomal) and dot-like [arrows] (integrated) HR-HPV DNA signals in high-grade dysplasia (CIN3) (CISH,200x) **J)** HPV16 DNA detection [arrows] in dysplastic epithelium (HPV16 ISH,200x); **K)** Binucleated atypical glandular cells (H&E stain,1000x); **L)** $p16^{INK4a}$ staining in endocervical glandular cells (IHC,400x); **M)** HPV L1 capsid protein detection in glandular cells (Viroaktiv® IHC, 1000x); **N)** HR-HPV DNA detection in glandular cells [arrows] (CISH,1000x); **O)** HPV31 DNA detection in cervical glandular cells [arrows] (HPV31 ISH,100x).

SUMMARY OF NEW FINDINGS

1. Our observations may promote better understanding of the epidemiology of HPV infections, especially regarding ones caused by multiple genotypes.

2. Our observations seem to validate the theory, that persistent infections of a single genotype play a primary role in the pathomechanism of cervical cancer and precancerous lesions. The topic of our study is current (Harald zur Hausen – Medical Nobel Prize 2008), its importance regarding people's health is paramount.

3. We have reflected upon the problematics of virus detection in the cases of multiple HPV infections by using molecular biological methods on cytological samples and in situ hybridization using histological samples.

4. We have shown the differences between the cummulative prevalance and the cross-sectional prevalance of single visits, and highlighted the importance of follow-up studies.

5. As far as we know, this is the first dinamic follow-up study of HPV DNA detection and genotyping within the setting of routine oncocytological screening regarding persistent HPV infections.

6. As far as we know, this is the first hungarian study that describes classical and non-classical cytomorphological signs of HPV infection at the histological level in cases of multiple HPV infections tested by molecular biological methods. Also, this is the first such study using immunohistochemistry and in situ hybridisation to detect the presence of HPV within both squamous and glandular epithelium and providing evidenced regarding the histological distribution of different genotypes in cases with multiple HPV infection.

LIST OF PUBLICATIONS

Original articels

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2. Abraham H, Veszpremi B, Gömöri E**, Kovacs K,** Kravjak A, Seress L. Unaltered development of the archi- and neocortex in prematurely born infants: genetic control dominates in proliferation, differentiation and maturation of cortical neurons. Prog Brain Res 164:3-22, 2007.
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Citable abstacts

Abraham H, Veszpremi B, Gömöri E, **Kovacs K**, Kravjak A, Seress L. Pre- and postnatal development of the calcium-binding protein-containing interneurons in the human cortex. IBRO Workshop, Clinical Neuroscience, Budapest, 2006.

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