Ph. D. THESIS

The prognostic role of human papillomaviruses (HPVs) in the precancerous lesions of the cervix uteri

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2004

INTRODUCTION

The cervical cancer is the third most frequent malignant tumor in women worldwide. The invasive cervix carcinoma is preceded by progressive dysplastic lesions, the *cervical intraepithelial neoplasia*s (CINs). The CIN lesions used to develop mostly in the third and fourth decade of life, nevertheless, nowadays the occurrence is shifted also to the age group 20-30. Since the development from cervical dysplasias to cervical cancer takes years, organized cervical screening can recognize most cervical lesions in the precancerous stage. The etiologic role of human papillomaviruses (HPVs) in the squamous cell neoplasias of the female genitalia is well established. From oncologic standpoint, the anogenital HPV types are classified into two groups. The HPV types 6, 11, 42, 43, 44 belong to the low-risk group and cause benign lesions and the high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) that can be detected in different grades of CIN lesions and in invasive cervical carcinoma. The incidence of the HPV infection is the highest in the age of 20-25 and declines with increasing age. The cervical HPV infection is most common in young, sexual active women. Since the HPV is a sexually transmitted pathogen, the infection rate is associated with sexual promiscuity, i.e. the number and the frequent change of sexual partners. The susceptibility for developing CIN is influenced also by previous sexually transmitted diseases and injuries of the uterine cervix. The cervical carcinomas of squamous cell origin carry HPV genome in nearly all of the cases; the viral genome is integrated usually into the host cell genome. The HPV type 16 and 18 are the most prevalent types in invasive cervical cancer worldwide. The HPV18 is frequently detected in adenocarcinomas while the HPV16 is frequently detected in all types of cervical carcinomas. The cervical cancers carrying HPV18 tend to progress faster and to have worse prognosis than those carrying HPV16. Other cancer associated types of HPV like type 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 are found in approximately one third of the cervical cancers and are frequent causes of precancerous cervical lesions. According to epidemiological data, also in the high-risk group, there are differences in the oncogenicity among the HPV types. HPV16, 18, 45, 52 and 59 infections have a stronger association with cervical carcinoma than the other types. For HPV type 16, the oncogenic potential further varies with intratype variants: an Asian-American variant has been reported to have a stronger cancer association than the European variants.

Nevertheless, only a small proportion of the high-risk HPV infections develops eventually into invasive cervical carcinoma. The course of HPV infection has an essential impact on the development of precancerous lesions and cervical cancer. In the majority of the cases –

especially under 30 years – the infections are transient, lesions of oncologic significance do not develop, the host eliminates the infected cells. In persistent infections, the E6 and E7 papillomaviral oncoproteins can induce dysplastic changes and can both initiate and maintain the development of precancerous lesions (CIN2, CIN3). The latters are characterized by dyskariotic changes and differentiational disorders of the squamous cells. Even the CIN3 may be reversible, although malignant conversion can also occur. This is the ultimate stage, at which cervical cancer may be prevented completely.

For patients with equivocal cytological results, the detection of the oncogenic HPV types is important to determine who are at risk of the developing lesions of oncogenic significance. In Hungary, the HPV detection is used as a secondary screening for patients with equivocal cytology. International studies investigate currently whether the HPV detection could be part of the primary screening as a supplement to cytology. If yes, cost-benefit calculation would still restrict primary HPV screening at age above 30-35 years, where the HPV incidence is low but the infection rather tends to persist. Under the age of 35 the occurrence of the HPV infection is more frequent but the infection is usually transient.

Although high-risk HPV infection is the major etiologic factor of cervical cancer, there are a great number of cofactors that may influence the outcome of the HPV infection. Preceding infections or co-infections with, for example, Chlamydia trachomatis, render the cervical epithelium more susceptible to HPV-related neoplasia. On the host side, there are also cofactors for cervical neoplasia such as smoking and high reproductive activity. In the normal transformation zone, from which most cervical neoplasias arise, there is increased expression of TGF-β1 and IL-10, which can suppress cell-mediated immunity locally. In high-grade CIN lesions, IFN-γ expression is decreased and IL-10 expression is increased. There are data concerning the possible role of the IL-10 promoter polymorphism in the malignancies of the uterine cervix.

The 5’ flanking region of the IL-10 gene is mapped at > 4 kb in length. In its proximal part, there are single nucleotide polymorphisms at nucleotide positions - 1082 (A/G), - 819 (C/T), and - 592 (C/A) relative to the transcriptional start. Because of a strong genetic linkage, there are three common haplotypes, GCC, ACC and ATA, in the Caucasian population. The number of the possible genotypes is six. The phenotype of the IL-10 producing ability can be determined by the nt-1082 polymorphism: low (AA), medium (AG) and high (GG) cytokine production.

AIMS

In the first study, we focused on the diagnostic and epidemiological significance of the human papillomavirus (HPV) genotyping within the high-risk group. Multicenter studies on the association between HPV types and *cervical carcinoma* indicated biological variance in the high-risk HPV group. Analyzing the clinical data and the virologic results we addressed the following issues:

- Is there a similar biologic variance within the high-risk HPV group in the development of precancerous *cervical intraepithelial neoplasia* to that found in invasive *cervical carcinoma*?
- Can multiple HPV infection increase the risk of *cervical intraepithelial neoplasia* in comparison with the risk exposure by single high-risk HPV infection?
- Can viral load data refine further the risk of *cervical intraepithelial neoplasia*?

In the second study we analyzed the association of IL-10 promoter nt - 1082 (A/G) polymorphism with the baseline data of HPV infection and cervical abnormalities and with the outcome of the cervical disease. Thus the all three nt-1082 genotypes were represented in sufficient number in contrast to previous studies. The clinical and virologic data were analyzed together with nt-1082 genotypes and the following issues were addressed:

- Can the IL-10 nt - 1082 polymorphism influence the susceptibility to high-risk HPV infection and cytological atypia?
- Can the IL-10 nt - 1082 polymorphism influence the risk of *cervical intraepithelial neoplasia*?

CLINICAL SAMPLES AND METHODS

Study groups

In a retrospective study examining the association of the high-risk human papillomavirus infection and the *cervical intraepithelial neoplasia*s (CINs), we identified 455 patients having had cervical HPV test due to cytological or colposcopic atypia between May 1997 and December 1999, and making consecutive visits after the HPV testing.

Digene's Hybrid Capture Tube test (Digene, Madison, MA) was used to detect the low-risk and the high-risk HPV infections. The follow-up data of the patients were collected from the electronic patient registry of the Medical Center. Time zero was set at the visit when the cytological or colposcopic atypia was first detected. The endpoint of the follow-up was the histological diagnosis of CIN determined in cervical lesions removed or the last registered visit.

In the study examining the association of the interleukine-10 (IL-10) promoter polymorphism, HPV infection and citological/colposcopic atypia we identified the firs 140 high-risk HPV positive and the firs 140 HPV-uninfected cervical specimens of the patients.

HPV genotyping

DNA isolated from the cervical Hybrid Capture specimens was subjected to the MY09-MY11 PCR amplification as described previously by our research group. The genotyping within the high-risk HPV group was done by RFLP analysis of the MY09-MY11 PCR products.

Viral load was assessed by the strength of hybridization signal in the Hybrid Capture test and was expressed in relative light units compared to 10 pg/ml HPV16 DNA positive control (RLU/PC).

IL-10 promoter nt-1082 typing

DNA isolated from the cervical Hybrid Capture specimens was used to analyze the polymorphic nt–1082 of the IL-10 promoter with allele-specific PCR. The 3' end of the sense allele-specific primers was complementary with the polymorphic nt-1082 while the antisense primer did not cover my polymorphic part. Both the A and the G specific PCR detection were performed in the samples.

Statistical analysis

We carried out univariate and multivariate Cox proportional hazards regression analyses to estimate the relative risk (RR) and 95% confidence intervals ($CIs_{95\%}$) of developing CIN according to the HPV infection, cytological alterations and the nt-1082 polymorphism.

Differences in discrete type data (e.g. nt-1082 polymorphism, cytological result, presence of HPV infection) between the patient groups was evaluated with Yates corrected chi square statistics. The susceptibility of the nt-1082 genotypes for the HPV infection and for the cytological atypia was tested by multivariate logistic regression analysis. The differences in continuous type data such as age distribution and viral load were tested with either two sample Kolmogorov-Smirnov statistics or multiple sample Kruskal-Wallis statistics.

RESULTS AND DISCUSSION

Role of the high-risk HPV types in the development of *cervical intraepithelial neoplasia* (CIN)

In the first study following-up 455 patients, we examined the occurrence of *cervical intraepithelial neoplasia*s (CINs) related to HPV infection and cervical abnormalities. High-risk and low-risk HPV infections were identified in 152 and 23 patients, respectively. The cervical specimens of 16 patients were positive in both the low-risk and the high-risk Hybrid Capture test. In the analysis of the Hybrid Capture results, the double positive patients were grouped together with the high-risk positives. The proportion of cervical atypia patients with only low-risk HPV infection (5%) was lower than that of the high-risk positives (37%).

The histological examination of the surgically excised biopsies revealed CIN grade 1 in 13 cases, CIN grade 2 in 31 cases, CIN grade 3 in 19 cases, carcinoma in situ in 23 cases, and microinvasive cancer in 2 cases. In the high-risk HPV infected cytological atypia group, CIN was detected in 77 (58,7%) of the 131 patients and 69 (52,6%) patients had high-grade lesions (CIN grade 2 or more severe). 88% of all CINs and 92% of the high-grade cases were detected in this group. The relative risk of the high-grade intraepithelial neoplasias (grade 2, grade 3, carcinoma in situ) was 16,2 (CI_{95}: 3,9-66,6) for P3 cytological atypia and 76,8 (CI_{95}: 23,7-249,5) for high-risk HPV infection.

The age distribution of the above study groups was different. Patients with cytological atypia were older than those without (median age: 34 vs. 28, p=0,001). In the cytological atypia group, the HPV uninfected patients were older than those with high-risk HPV infection (median age: 38 vs. 31, p<0,001). The effect of age on developing CIN was computed together with high-risk HPV infection and cytological atypia. The relative risk of the high-grade intraepithelial neoplasias was 1,99 ($CI_{95\%}$: 1,26-3,16) for patients above 35 years compared to patients below 35.

Of the 168 specimens found high-risk HPV positive in the Hybrid Capture test, 150 (89,2%) were genotyped by PCR-RFLP and were used to evaluate differences in oncogenic potential within the high-risk group of HPVs. Genotyping revealed that co-infections with multiple HPV types were present in 19 (12,7%) patients. The relative risk of high-grade CIN following multiple high-risk HPV infection was lower (RR: 49,5; CI_{95}: 12,2-201,7) but did not differ significantly from the infections caused by single HPV type (RR: 85,6;CI_{95}: 26,2-279,3). Taking both the single and the multiple infections into account, the most common types were

HPV16, HPV33, HPV31, and HPV18, which were found in 78 (52%), 21 (14%), 20 (13,3%), and 9 (6%) cases, respectively. Less common high-risk types (HPV35, HPV45, HPV51, HPV52, HPV56, HPV58) were present in 26 (17,3%) cases. Due to the limited size of the study, the high-risk genotypes were grouped as follows: 1.: HPV16/18, 2.: HPV45/52/56, 3.: HPV31/33/35/51/58. Among the patient groups classified by the different high-risk HPV types, the differences in age distribution (p=0,57) and in the frequency of cytological atypia (p=0,63) were not significant. The multivariate estimates of the relative risk revealed that all high-risk genotypes were significant risk factors for CIN. We identified HPV16 or HPV18 type in 87 cases. CIN developed in 57 cases (RR: 52,7; CI_{95}: 24,2-114,8) of which CIN grade 2 or worse were 51 cases (RR: 119,1 CI_{95}: 36,2-390,9). The highest risk factor values were revealed in this group. The HPV31/33/35/51/58 group consisted of 43 patients, CIN developed in 12 patients (RR: 17,2; CI_{95}: 6,9-43,2) of which CIN grade 2 or worse were in 11 cases (RR: 39,7; CI_{95}: 10,9-144,8). The HPV45/52/56 group consisted of 11 cases, CIN developed in 4 cases (RR: 17,8; CI_{95}: 5,3-60,5) of which CIN grade 2 or worse were in 4 cases (RR: 44,4; CI_{95}: 9,8-201,0). Among the HPV groups, the HPV16/18 group had approximately three times higher oncogenic risk than the less common cancer associated types.

As expected, both high-risk HPV infection and atypical cytology were major risk factors for having CIN or worse histological diagnoses. Older age (>35) proved to be also an independent risk factor. Consistently, most but not all high-grade CINs were detected in the high-risk HPV positive cytological atypia group and all high-grade CINs were predicted by either cytological atypia or high-risk HPV infection.

Multiple HPV infections did not confer any increased oncogenic risk over single high-risk infections. In fact, the point estimates of the relative risk of CIN after multiple infections were lower than after single infections. We found approximately three times higher oncogenic risk in the HPV16/18 group than the less common cancer associated types. The HPV45/52/56 group did not prove to have higher risk for the CIN than the group of other less common types.

The viral load was assessed by the strength of the hybridization signal (RLU/PC) measured in the Hybrid Capture test. This estimate of the viral load was associated with neither cytological atypia (p=0,87) nor age above 35 (p=0,77) nor the different groups of high-risk types (p=0,70). Neither the high viral load (>100 RLU/PC; RR: 0,86; CI_{95}: 0,33-2,23) nor the medium viral load (10-100 RLU/PC; RR: 1,54; CI_{95}: 0,95-2,51) had an increased oncogenic risk for high-grade CIN compared to the low viral load (1-10 RLU/PC). According to our

results we can not draw further prognostic conclusions from the viral load data in patients selected by cytological or colposcopic disorders during the primer screening.

The role of the IL-10 nt-1082 polymorphism in the cervical carcinogenesis

In the second study using the nt-1082 allele-specific PCR methodology, IL-10 promoter-specific sequences could be amplified from 125 high-risk HPV-positive and 128 HPV-negative cervical abnormalities. At the polymorphic nt-1082 of the IL-10 promoter, the frequency distribution of the genotypes of the control group were AA: 25%, AG: 51%, GG: 24%, which showed good fit to the Hardy-Weinberg equilibrium (p=0,95). The A and G allele frequencies were 0,51 and 0,49, respectively. Among the patients with cervical abnormalities, the allele-specific PCR analysis revealed an altered frequency distribution of genotypes (p=0,05): AA genotype was detected in 88 cases (35%), AG genotype in 123 cases (49%), and GG genotype in 42 cases (16%). However, the alteration was not evenly shared between the HPV positive and the HPV uninfected patient groups. The frequencies of AA (28%), AG (52%) and GG (20%) genotypes in the HPV positive group were not significantly different from those of the controls (p=0,70). On the other hand, the nt-1082 allele frequencies were altered among the patients with HPV negative cervical abnormalities (A: 0,66; G: 0,34). With regard to the genotypes, the frequency distribution in patients with HPV negative normal cytology were the following: AA: 40%; AG: 42%; GG: 18% while the frequency distribution of the genotypes were differed significantly from that of the controls in patients with HPV negative equivocal cytological atypia: AA: 42%; AG: 47%; GG: 11%; (p=0,006).
In the analysis of the disease association of the nt-1082 genotypes, we took into account that the A allele is much more frequent than the G allele in most non-Caucasian populations. Therefore, AA was regarded as the reference genotype, and the relative disease association of AG and GG genotypes was expressed by calculating the odds ratios (OR) and their 95% confidence intervals. The chance of the patients with GG genotype for the HPV negative cytological atypia (P3) was significant less (OR: 0,27; CI_{95}: 0,11-0,63) than in patients with AA genotype, a borderline significance was revealed in patients with AG genotype (OR: 0,56; CI_{95}: 0,31-1,02). The distribution of nt-1082 genotypes was not different between the older (>35) and the younger (<35) patients, as using the AA genotype as a reference, the ORs for AG and GG were 1,18 (CI_{95}: 0,71-1,97) and 0,78 (CI_{95}: 0,41-1,50), respectively.

In conclusion, we found no association between IL-10 nt-1082 polymorphisms and HPV-related cervical abnormalities, which indicates that this polymorphism does not influence the HPV infection itself.

The outcome of the baseline cervical abnormalities was evaluated longitudinally during a follow-up. Altogether, 53 high-grade CIN cases were identified, of which 50 were detected in patients with high-risk HPV positive equivocal cytological atypia. Relative risks (RR) for high-grade CIN were calculated. As expected, high-risk HPV infection (RR: 104, 6; CI_{95}: 14,2-769,9) and the presence of equivocal cytological atypia (RR: 9,6; CI_{95}: 2,3-39,6) were independent risk factors for high-grade CIN. The relative risks also did not reveal any significant difference between the IL-10 nt –1082 genotypes, AA: 1,0; AG: 1,11 (CI_{95}: 0,59-2,08); GG: 0,62 (CI_{95}: 0,25-1,05) indicating that this polymorphism does not influence the progression to high-grade CIN.

In conclusion, the patients with different nt-1082 genotypes did not have different risks for developing precancerous cervical lesions. Thus, IL-10 promoter nt-1082 polymorphism was not found to be a cofactor in the early stage of cervical carcinogenesis.

On the other hand, women carrying the nt-1082 G allele in the IL-10 promoter demonstrated less susceptibility to cytological atypia unrelated to HPV infection, which is, anyhow, not associated with increased oncogenic risk. The increased IL-10-producing ability encoded by the nt-1082 G allele can protect against cytological atypias, which develop due to the inflammation.

SUMMARY

The differences in the oncogenity of the high-risk human papillomaviruses (HPVs) genotypes were investigated in a hospital-based study. The relative risk of the high-grade intraepithelial neoplasias (CIN2, CIN3, carcinoma in situ) were 16,2 (CI_{95}: 3,9-66,6) for P3 cytological atypia, 76,8 (CI_{95}: 23,7-249,5) for high-risk HPV infection and 1,99 (CI_{95}: 1,26-3,16) for the age above 35. Multiple HPV infections were detected in 12,7%, and its relative risk (RR: 49,5; CI_{95}: 12,2-201,7) did not differ significantly from that of single type HPV infections (RR: 85,6;CI_{95}: 26,2-279,3). The high-risk HPV genotypes were grouped in three different groups as follows: 1.: HPV16/18, 2.: HPV45/52/56, 3.: HPV31/33/35/51/58. The relative risk in the first group was 119,1 (CI_{95}: 36,2-390,9), in the second group, it was 44,4 (CI_{95}: 9,8-201,0), and in the third group it was 39,7 (CI_{95}: 10,9-144,8). The ratio of the relative risks among the types occurring in the invasive carcinomas did not exceed three fold value, which make the further genotyping in the high-risk HPV group unnecessary from the point of diagnosis and prognosis.

Viral load was assessed by the strength of hybridization signal and was adjusted to the DNA content of the sample. Viral load above the detection limit of Hybrid Capture HPV test showed no association with CIN. The viral load was also independent of the other examined risk factors, the HPV genotype, presence of cytological atypia, age.

In addition to the high-risk HPV types, the risk of CIN may be influenced by host and other microbiological cofactors. On the host's part we examined the role of the IL-10 promoter nt-1082 polymorphism (nt-1082 A/G). The frequency distribution of the nt-1082 genotypes in the HPV related cytological atypia was not different from the control group (p=0,70) and it did not influence the risk of CIN (AA: ref, AG: RR=1,11 [CI_{95}: 0,59-2,08], GG: RR=0,62 [CI_{95}: 0,25-1,50]). On the other hand the non-AA genotypes characterized by higher IL-10 producing ability had less susceptibility to the HPV negative cytological atypia (AA: ref. AG=0,56 [CI_{95}: 0,31-1,02], GG=0,27 [CI_{95}: 0,11-0,63]). In conclusion, the nt-1082 polymorphism had no influence on the early phase of cervical carcinogenesis but may determine different susceptibilities to cervical abnormalities unrelated to HPV infection.

PUBLICATIONS

This thesis is based on the following publications:

Krisztina Szőke[1], Tamás Sápy[2], Zoárd Krasznai[2], Zoltán Hernádi[2], Györgyi Szládek[1], György Veress[1], Joakim Dillner[4], Lajos Gergely[1, 3] and József Kónya[1]*
Moderate variation of the oncogenic potential among high-risk human papillomavirus types in gynecologic patients with cervical abnormalities. *Journal of Medical Virology*
2003. Dec; 71 (4): 585-92. (IF: 2,629)

Krisztina Szőke[1] Anita Szalmás[1] Györgyi Szládek[2] György Veress[1] Lajos Gergely[1,2] Ferenc D. Tóth[1] and József Kónya[1]*
Interleukin-10 promoter nt-1082A/G polymorphism and human papillomavirus infection in cytologic abnormalities of the uterine cervix. *Journal of Interferon & Cytokine Research* (in press) (IF: 1,885)

Other publications:

Krisztina Szőke, Györgyi Szládek, Krisztina Szarka, Attila Juhász, György Veress, Lajos Gergely, József Kónya
Human cytomegalovirus load in the peripheral blood determined by quantitative competitive polymerase chain reaction. *Acta Microbiologica et Immunologica Hungarica,* 2001.; 48 (3-4): 313-21.

Györgyi Szládek, Attila Juhász, László Asztalos, **Krisztina Szőke**, Melinda Murvai, Krisztina Szarka, György Veress, Lajos Gergely, József Kónya
Persisting TT virus (TTV) genogroup 1 variants in renal transplant recipients. *Arch. Virol*., 2003. May; 148 (5): 841-51. (IF: 1,967)

ORAL PRESENTATIONS, POSTERS

Szőke K., Szládek Gy., Hernádi Z., Gergely L., Kónya J.: Humán papillomavírus (HPV) kimutatás cervicalis intraepithelialis neoplasia (CIN) műtéti eltávolítása után (Detection of

human papillomavirus (HPV) following surgical excision of the cervical intraepithelial neoplasia (CIN))
Jubilee Congress of the Hungarian Society for Microbiology, October 10-12., 2001. Balatonfüred (presentation)

Kónya J., **Szőke K**., Szládek Gy., Hernádi Z., Gergely L.: A virológiai státusz epidemiológiai és diagnosztikai jelentősége onkogén humán papillomavírus (HPV) fertőzésben (The epidemiologic and diagnostic significance of the virological status in oncogenic human papillomavirus (HPV) infection

Jubilee Congress of the Hungarian Society for Microbiology, October 10-12., 2001. Balatonfüred (presentation)

Szőke K., Kónya J., Szarka K., Veress Gy., Juhász A., Gergely L.: Humán cytomegalovírus DNS mennyiségi meghatározása polimeráz láncreakcióval (Human cytomegalovirus load in the peripheral blood determined by quantitative competitive polymerase chain reaction)
First Joint Meeting of the Slovenian Society for Microbiology and the Hungarian Society for Microbiology, August 24-26.,2000., Keszthely (poster)

Szőke K., Szládek Gy., Gergely L., Kónya J.: Distribution of high-risk HPV types by grading of cytologic atypia and development of high grade CIN
20th International Papillomavirus Conference, October 4-9., 2002., Paris (poster)

Kónya J., Sápy T., **Szőke K.,** Szládek Gy., Hernádi Z., Gergely L.: Predictive value of human papilloma virus (HPV) testing for incident and recurrent cervical intraepithelial neoplasia (CIN)
20th International Papillomavirus Conference, October 4-9., 2002., Paris (poster)

Szőke K., Szládek Gy., Gergely L., Kónya J.: Role of interleukine-10 (IL-10) promoter polymorphism in premalignant lesions of the uterine cervix

14th International Congress of the Hungarian Society for Microbiology, October 9-11., 2003., Balatonfüred (poster)