Incidence and association of the human papilloma virus (HPV) and four selected bacterial sexually transmitted pathogens with normal, non-neoplastic and neoplastic cervical cytology

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In this work, we analysed the incidence of the human papilloma virus (HPV) and four sexually transmitted bacterial pathogens (bSTD; *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma hominis, Ureaplasma urealyticum)* in 529 cervical samples from Slovakian women and evaluated its association with normal, non-neoplastic and neoplastic cervical cytology. The non-neoplastic cervical samples represented the largest (71%), while the normal cervical cytology samples the smallest (9%) group of investigated specimens. The majority of women with a normal or neoplastic cervical cytology were 29 years old or younger, in contrast to women with non-neoplastic cervical cytology, who were mostly older. The 50 year-old or older women represented the smallest group of patients with non-neoplastic (8%) and neoplastic (5%) cervical cytology, while none of these women was found with a normal cervical cytology. Women with normal cervical cytology were statistically significantly more likely to have a cervical HPV infection than women with non-neoplastic cervical cytology. On the other hand, women with neoplastic cervical cytology were significantly more likely to have a cervical HPV (and combined HPV/bSTD) infection than women with normal or non-neoplastic cervical cytology. Finally, *Ureaplasma* was found to be the most prevalent bSTD pathogen. Thus, these results are in good concordance with the published literature, however, analyses of new (genetically) defined abnormalities and/or new approaches are needed to improve the screening and diagnostics strategies of cervical pathology.
Keywords: cervical cytology, human papilloma virus (HPV), incidence, sexually transmitted disease (STD), statistical significance

Výskyt a spojitosť ľudského papilomavírusu (HPV) a štyroch vybraných sexuálne prenosných bakteriálnych patogénov s normálnou, nonneoplastickou a neoplastickou cervikálnou cytológiou
V uvedenej práci sme analyzovali výskyt ľudského papilomavírusu (HPV) a štyroch vybraných sexuálne prenosných bakteriálnych patogénov (bSTD; Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma hominis, Ureaplasma urealyticum) v 529 cervikálnych vzorkách slovenských žien a hodnotili sme ich spojitosť s normálnou nonneoplastickou a neoplastickou cervikálnou cytológiou. Najväčšiu skupinu vyšetrovaných vzoriek (71 %) predstavujú nonneoplastické vzorky, zatiaľ čo tou najmenšou (9 %) sú vzorky s normálnou cytológiou. Väčšina žien s normálnou alebo neoplastickou cervikálnou cytológiou je vo veku 29 rokov alebo mladšia na rozdiel od žien, väčšinou starších, s non-neoplastickým výsledkom cervikálnej cytológie. Ženy vo veku 50 a viac rokov reprezentujú najmenšiu skupinu spomedzi žien s nonneoplastickou (8 %) alebo neoplastickou (5 %) cytológiou, zatiaľ čo žiadna žena s normálnym výsledkom cervikálnej cytológie nemá 50 a viac rokov. Ženy s normálnou cervikálnou cytológiou mali štatisticky častejšie HPV infekciu v porovnaní so ženami s nonneoplastickou cytológiou. Na druhej strane ženy s neoplastickou cervikálnou cytológiou boli štatisticky významne viac postihnuté HPV (a kombinovanou HPV/bSTD) infekciou ako ženy s normálnou alebo nonneoplastickou cervikálnou cytológiou. Navyše, *Ureaplasma* bola v tejto štúdii zachytená ako najčastejší bSTD patogén. Tieto výsledky sú vo všeobecnosti v dobrom súlade s publikovanou literatúrou, avšak analýzy nových (genetických) abnormalít a/alebo nové prístupy sú potrebné na vylepšenie skríningových a diagnostických stratégií cervikálnej patológie.
Kľúčové slová: cervikálna cytológia, ľudský papilomavírus (HPV), incidencia, sexuálne prenosné ochorenia (STD), štatistická významnosť

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Introduction

Cervical cancer is the second most common cancer in women worldwide and one of the leading causes of cancer-related death among women in developing countries (see for example 1 and references therein). Cervical cancer mostly occurs in the proliferation region between the columnar epithelium of the endocervix and the stratified squamous epithelium of the exocervix. Careful cervical cytology monitoring stays our basic tool in prevention of cervical neoplasia[2].

The carcinogenic human papilloma virus (HPV) is the most important risk factor of cervical cancer (see for example 3 and references therein). Oncogenicity in tumours caused by HPV is due to the prevalence of early genes E6 and E7 which disrupt normal cell growth and inhibit tumour-suppressor proteins (for review see for example 4). The HPV infection might lead to a latent co-existence of HPV with a host over long periods of time. Immune suppression in humans usually leads to activation of latent infection and to development of various non-neoplastic or neoplastic lesions (for review see for example 5). However, the extended time gap between initial HPV infection and onset of cancer and a relatively minor fraction of infection resulting in neoplasia suggests that there are other factors that contribute to malignant progression of the initial lesions. Interesting candidates for such factors are sexually transmitted disease (STD) bacteria like *Chlamydia trachomatis*, *Neisseria gonorrhoeae, Mycoplasma hominis* or *Ureaplasma urealyticum*, which are often associated with genital and mucosal HPV infection and might therefore act in synergy with HPV to induce neoplasia[6-9]. In this work, we analysed the incidence of HPV and four latter bacterial STD pathogens in 529 cervical samples and evaluated its association with normal, non-neoplastic and neoplastic cervical cytology.

Patients and methods

In this study, we analysed cervical samples of 529 women attending local gynaecology ambulances in the Presov area of East Slovakia, between 2009 and 2012. Cervical samples were collected with a Dacron swab. One part of the cervical sample provided smears, which were subjected to cytological investigation in the CytoLab, s. r. o. laboratory in Presov. Each cytological diagnosis was established according to the 2001 Bethesda system criteria[2] and investigated cervical samples were classified into the three cytology groups: a.) normal cervical cytology; b.) non-neoplastic cervical cytology (involves inflammation, atrophy, reactive cellular changes associated with inflammation) and c.) neoplastic cervical cytology (involves atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL)). Another part of the cervical material was subjected to isolation of DNA and to genetic analyses in the SEMBID, s. r. o. laboratory in Kosice. HPV DNA of carcinogenic risk HPV genotypes [16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59] was detected using the AmpliSens HPV HCR screen-titre-FRT PCR kit according to manufacturer instructions. The nested PCR protocols were used to detect the *Neisseria gonorrhoeae*[10] and *Chlamydia trachomatis*[11] DNA, while the single PCR protocols were used to detect the *Mycoplasma hominis* and *Ureaplasma urealyticum*[12] DNA. Statistical analyses were performed using a chi-square test at www.socscistatistics.com. A *P* value of <0,05 was deemed statistically significant.

Results

Age-dependent incidence of different cytology results in 529 cervical samples

From the 529 cervical samples involved in this study we detected normal cytology in 46 (9%) cases; non-neoplastic cytology in 374 (71%) cases, while the neoplastic cytology results were found in 109 (20%) of analysed specimens ***(Table 1)***. The age-dependent incidence of patients within the investigated normal, non-neoplastic and neoplastic cytology is presented in ***Table 1***.

Detection of HPV and selected bacterial pathogens in samples with normal, non-neoplastic and neoplastic cytology

Next, we investigated the 529 cervical samples, described above, for presence or absence of DNA from HPV and the four bacterial STD-pathogens (bSTD) *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma hominis* and *Ureaplasma urealyticum*. The sample containing DNA from HPV and at least the one bacterial pathogens, described above, was depicted as HPV- and bSTD-positive, respectively, while the sample containing both the HPV DNA and DNA from at least one bacterial pathogen was depicted as HPV/bSTD-positive. All other samples were described as negative. ***Table 1*** provides a summary of this analysis.

When we compared the age-dependent incidence of the investigated HPV and bSTD positive or negative cervical

Table 1. *Incidence of HPV and four selected bacterial STD (bSTD) pathogens in 529 cervical samples with different cytology*

Patient age	negative	HPV+	bSTD+	HPV/ bSTD+	total	total (%)
Cervical samples with normal cytology						
≤ 29 y	5	**9**	**2**	**6**	22	48
30-39 y	**13**	2	1	2	18	39
40-49 y	5	1	0	0	6	13
≥ 50 y	0	0	0	0	0	0
total	23	12	3	8	46	100
total (%)	50	26	7	17	100	
Cervical samples with non-neoplastic cytology						
≤ 29 y	47	28	19	**14**	108	29
30-39 y	**85**	**33**	**23**	11	152	40
40-49 y	55	10	13	7	85	23
≥ 50 y	20	5	2	2	29	8
total	207	76	57	34	374	100
total (%)	56	20	15	9	100	
Cervical samples with neoplastic cytology						
≤ 29 y	**16**	**20**	**4**	**18**	58	53
30-39 y	11	10	**4**	7	32	29
40-49 y	7	4	2	1	14	13
≥ 50 y	3	2	0	0	5	5
total	37	36	10	26	109	100
total (%)	34	33	9	24	100	

The highest number of cervical samples within the particular analysed negative, HPV positive (HPV+), bSTD positive (bSTD+) and HPV/bSTD positive (HPV/bSTD+) group are depicted in bold

samples in the three different cytology groups, described above, the majority of the normal cytology samples with bSTD-, HPV- or HPV/bSTD- positivity were derived from women younger as 29 years of age, in contrast to the negative samples, of which the majority were derived from women between 30-39 years of age ***(marked in bold in Table 1)***. Similarly, the majority of patients with non-neoplastic cervical cytology and the HPV/bSTD-positivity were younger than 29 years of age, in contrast to the three other groups which were mostly derived from women between 30-39 years of age ***(marked in bold in Table 1)***. Finally, as described above, the majority of patients with neoplastic cervical cytology with or without HPV- and/or bSTD-positivity were younger than 29 years of age ***(marked in bold in Table 1)***.

Association between cytology and various other parameters of investigated cervical samples

Table 2 presents the chi-squared test analysing the association between the cytology results and the age-, HPV- and bSTD-profiles of the normal versus non-neoplastic cervical cytology samples (the first panel from the left), the normal versus neoplastic cervical cytology samples (the second panel from the left) and the non-neoplastic versus neoplastic cytology samples (the third panel from the left).

Discussion

In this work, we used the cervical cytology and genetics (see Patients and methods) to analyse the age-dependent incidence of HPV and four selected bacterial STD pathogens (*Chlamydia trachomatis*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Neisseria gonorrhoeae*) and to evaluate its association with normal, non-neoplastic and neoplastic cytology in a group of 529 cervical samples.

The non-neoplastic cervical cytology samples represent the largest (71%), while the normal cervical cytology samples the smallest (9%) group of samples investigated in this study. A low number of women in this study with a normal cytology result probably indicates that women attending gynaecology ambulances for a preventive check-up are in the minority. In addition, no woman of 50 years or older was found in the latter group ***(Table 1)***, indicating that older women are probably almost absent in the gynaecologic prevention program of cervical cancer. However, more data are needed to better explore this speculation.

Curiously, HPV DNA was found in 43% of normal cervical samples but in only 29% of non-neoplastic cervical samples often associated with inflammation (see Patients and methods). This somehow protective association between a positive HPV status and a decreased risk for non-neoplastic cervical lesions might be of an artificial origin, due to a low number of cervical samples with normal cytology in this analysis. However, more analyses are needed to explore this option in more detail. On the other hand and as expected (despite a low number of cervical samples with normal cytology), neoplastic cervical samples had statistically a significantly higher risk profile for HPV infection that other cervical samples ***(Tables 1, 2; see for example 1,13 and references therein)***. Moreover, a majority of HPV positive women with normal and neoplastic cytology were up to 29 years of age ***(Table 1)***, which supports a previously documented tendency of HPV to preferentially appear in younger patients close to the age of sexual debut (9 and references therein).

At least one of four bacterial STD pathogens selected in the present study, was found in 33%, 24% and 24% of women with neoplastic, normal and non-neoplastic cervical cytology, respectively ***(Tables 1,2)***. However, the latter increase in neoplastic versus normal/non-neoplastic samples was not statistically significant ***(Table 2)***. The co-infection of HPV and at least one of the four bacterial STD pathogens selected in the present study, was found in 17% and 9% of women with normal and non-neoplastic cervical cytology, respectively, while a statistically significantly higher occurrence (24%) was found in women with neoplastic cervical cytology ***(Tables 1,2)***. The reported higher co-incidence of HPV and bacterial STD pathogens in neoplastic cervical samples here is fully in agreement with previous studies (see for example 8,9).

Table 2. *Chi-square analysis of different parameters of cervical samples with normal (N) and non-neoplastic (NNE), N and neoplastic (NE) and NNE and N cytology*

	cytology								
	N (%)	NNE (%)	P*	N (%)	NE (%)	P*	NNE (%)	NE (%)	P*
Patient age									
≤ 29 y	22(48)	108(29)		22(48)	58(53)		108(29)	58(53)	
30-39 y	18(39)	152(40)		18(39)	32(29)		152(40)	32(29)	
40-49 y	6(13)	85(23)	**0,027**	6(13)	14(13)	0,451	85(23)	14(13)	**0,003**
HPV									
positive	20(43)	110(29)		20(43)	62(57)		110(29)	62(57)	
negative	26(57)	264(71)	**0,039**	26(57)	47(43)	**0,047**	264(71)	47(43)	**0,00006**
bSTD									
positive	11(24)	91(24)		11(24)	36(33)		91(24)	36(33)	
negative	35(76)	283(76)	1	35(76)	73(67)	0,158	283(76)	73(67)	0,158
bSTD**									
Ct	3(18)	17(17)		3(18)	6(15)		17(17)	6(15)	
Mh	4(23)	17(17)		4(23)	6(15)		17(17)	6(15)	
Uu	10(59)	64(65)	0,545	10(59)	26(67)	0,298	64(65)	26(67)	0,259
HPV+bSTD									
positive	8(17)	34(9)		8(17)	26(24)		34(9)	26(24)	
negative	38(83)	340(91)	0,092	38(83)	83(76)	0,220	340(91)	83(76)	**0,004**

**Data with p < 0,05 are shown in bold types*

Finally, the *Ureaplasma urealyticum* was found to be a most prevalent bacterium from four investigated bacterial STD pathogens (*Chlamydia trachomatis*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Neisseria gonorrhoeae*; ***Table 2***), in line with the published literature (for review see 7). On the other hand, in contrast to the previously documented association between *Chlamydia trachomatis* and cervical neoplasia[8,14-17], we did not find any specific association between the individual investigated bacterial pathogen and the sample cytology ***(Table 2)***. This was probably due to a small number of samples in this study; but more analyses are needed to explore it in more detail. However, in this respect we note also some previous studies which did not observe a direct causal association between *Chlamydia trachomatis* and cervical neoplasia[18,19], or suggested only an increasing susceptibility to carcinogenic HPV infection among women with a positive *Chlamydia trachomatis* status[9].

Conclusion

Despite the study limitations, described above, we have found here a good congruence with the published literature on the incidence and association of HPV and HPV/bSTD pathogens with cervical pathology ***(see Tables 1, 2 and text above)***. This approach helps us to identify patients with higher risk for cervical pathology (i.e. persistent carcinogenic HPV or carcinogenic HPV/bSTD DNA positivity) and to involve them in more accurate medical care (see for example 13). However, we find here a relatively high portion of women with an HPV and/or HPV/bSTD negative result on the one hand and a neoplastic cervical cytology on the other hand ***(Table 1 and text above)***. Similarly, the recent paper by Tracht et al. also reported that the HPV-negative samples with positive cytology exist and may be missed by primary HPV screening[20]. Thus, the analysis of other defined differences (i.e. ethnicity; see for example 1) in women attending gynaecology ambulances and/or completely new approaches are needed to improve the screening and/or diagnostics strategies of cervical pathology. Our group is trying to explore this suggestion by a complex metabolomic approach using the fluorescent concentration matrix technology[21].

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