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ORIGINAL ARTICLE

Impact of combined mycoplasmataceae and HPV co-infection on females with cervical intraepithelial neoplasia and carcinoma

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Summary

Purpose: *The concurrent prevalence investigation of human papillomavirus (HPV), Mycoplasma hominis (Mh) and Ureaplasma urealyticum (Uu) in women in order to estimate the association of co-infection with cervical lesions.*

Methods: *The study cohort comprised 120 women with no cervical lesions (control group) and 62 women with abnormal cytological findings from the cervix (cervical intraepithelial lesion/neoplasia) as study group. A combination of molecular analyses was implemented.*

Results: *The presence of HPV infection was shown in 52/62 (83.9%) of women with abnormal cytology. Women with cervix cytological findings were shown to have 17.6 times higher risk for Mh and Uu co-infection (p=0.001). HPV and Uu co-infection were detected with a higher prevalence among women with CIN 3 and invasive cancer.*

Conclusion: *These findings are consistent with the notion that microbial co-infections may play an important role in persistent inflammation and progression of cervical lesions.*

Key words: *HPV typing, mycoplasma, ureaplasma, cervical lesions, carcinoma, greek population*

Introduction

Several epidemiological and molecular studies have revealed that human papillomaviruses (HPVs) are the main etiological agents in cervical carcinogenesis, since HPV types have been detected in almost all cervical cancer biopsies [1]. Moreover, type-specific HPV persistence is the strongest risk factor for cervical neoplasia and women infected with high-risk HPV types are considered to be at higher risk for developing cervical cancer [2-6]. While most sexually active women are infected at least once in their lifetime, many reports have shown that HPV infections are mostly transitory and that only a small percentage of HPV infections persist and may progress to malignancy [7-9]. Other risk factors, such as environmental factors including the concomitant infection with other microorganisms, have also been investigated as potentially involved in the disease process, by either preventing the spontaneous clearance of HPV or intensifying cervical inflammation leading to serious cervix pathology [10-12].

The prevalence of both HPV and other sexually transmitted microorganisms in a population mostly depends on the multiplicity of sexual partners. The association of concomitant infection with microorganisms, such as *Ureoplasma urealyticum,*

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Mycoplasma genitalium, Chlamydia trachomatis and *Trichomonas vaginalis,* with cervical dysplastic and neoplastic lesions has been demonstrated, although their specific role in the pathogenesis of cervical cancer has not been totally clarified [13-15]. The objective of the present study was to investigate the prevalence of different HPV types in gynaecologic malignant lesions, and to evaluate the existence of co-infection with HPV and other genital microorganisms: *Mycoplasma hominis (Mh) and Ureaplasma urealyticum (Uu)* in females with and without cervical lesions [16,17].

Methods

Clinical specimens

Vaginal specimens were collected from two groups of female patients, namely the control group and the target population group. The control group consisted of 120 asymptomatic women (age range 14-55 years; mean age 29.8 years) who attended the outpatient clinic of the 1st University Department of Gynecology of 'Alexandra' Hospital in Athens for a routine Pap test and/or for genital infection symptoms. The target population group consisted of 62 women (age range 19-79 years; mean age 40.7) who were referred to the Colposcopy and Laser Surgery Unit of Alexandra Hospital with abnormal Pap smears. The target group patients underwent a full colposcopy and cervical biopsies were obtained. Histologic examination classified the biopsies as follows: one normal, three with cervicitis, two with condyloma, twenty with HPV infection, seven with CIN 1, twelve with CI N 2/3 and seventeen with cervical carcinoma.

A brief gynecological history was obtained from all studied women who gave their informed consent to participate in the study. The protocol had been previously approved by the institutional ethics committee.

Culture procedures

Women from both groups were examined for the presence of *Mh* and *Uu.* Clinical samples were collected with a swab in Urea-Arginine LYO 2 broth (Biomerieux) containing 3 ml of genital mycoplasma transport medium. They were placed on ice and immediately sent to the laboratory. As soon as they arrived, the samples were inoculated in MycoTest (Bioprepare) consisting of urea broth (mycoplasma broth base, horse serum, yeast extract, isovitalex, urea, phenol red and antibiotic mixture pH 5.8) for *Uu* isolation and arginine broth (mycoplasma broth base, horse serum, yeast extract, isovitalex, arginine, phenol red and antibiotic mixture pH 6.1) for isolation of *Mh.* An aliquot of the original sample was inoculated onto A7 agar for culturing of both genital mycoplasmas. Urea and arginine broth cultures were incubated at 37°C in aerobic conditions and observed for a pH change within 48 h. The A7 agar plates were incubated at 37°C in an atmosphere of 5% CO_2 and examined microscopically daily for 5 days for the appearance of typical mycoplasma colonies.

Sample preparation for bacterial DNA and PCR

In some instances, PCR was performed in order to confirm discrepant *Mh* and *Uu* culture analysis. DNA was extracted from clinical specimens using Nucleospin Microbial DNA kit (Macherey, Nagel, Germany). The samples then were stored at – 20°C until PCR assay. Primers U4 and U5 were used for the urease gene of *Uu*, RNAH1 and RNAH2 for the 16S rRNA gene of *Mh*, respectively [18]. An internal control using primers to amplify a 262 bp fragment of the β-globin gene was used for PCR performance. The results of *Mycoplasma* DNA amplification were considered only when DNA was found to be positive for the internal control. Samples negative for the internal control and *Mycoplasma* amplification were not taken into account and were classified as indeterminate.

Detection of HPV DNA and genotyping

Colposcopy taken biopsies were immediately placed in sterile tubes with RNAlater™ solution (Ambion Inc., USA) and stored at –20°C until processed for DNA extraction. Total cellular DNA was isolated from biopsies using the Nucleospin TM DNA isolation kit (Macherey-Nagel, Germany). DNA from specimens were amplified for HPV DNA by polymerase chain reaction (PCR) with the consensus primers MYO9/MY11 located at the L1 open reading frame of several HPV types [19]. A positive and negative control weres used in each amplification reaction. The PCR products were analyzed by electrophoresis on 2% agarose gel, and visualized under ultraviolet transilluminator. HPV typing was performed by restriction fragment length polymorphism (RFLP) analysis, after digesting the amplification products separately, with the restriction endonucleases *Dde, Hinf1, Pst1, Rsa1* (Takara, Japan), as previously described [5]. The digested products were analysed by 3% Metaphor TM (Bio Whittaker Molecular Applications, U.S.A) agarose gel electrophoresis and the HPV types were determined according to previously published restriction patterns [20]. Nested PCR was used for detection of HPV DNA in samples with low viral load, as previously described [19]. Additionally, accuracy of HPV typing was confirmed by DNA sequencing of several samples (MACROGEN, Korea). All nucleotide sequences were searched in GenBank and each of them was considered a match if it was found to have more than 80% nucleotide similarity to a known HPV type sequence, as previously suggested [21].

Statistics

Statistical comparisons were performed using SPSS version 21.0 software package (IBM Corp., Armonk, NY, USA). Student's t-test was applied to assess age difference between women with no cytological findings (controls) from the cervix and those with any cytological finding. Two-tailed Fisher's exact test was used in order to assess possible differences in the prevalence rates of genital microorganisms under study between the two study groups. The same test was also performed to assess possible association between HPV presence and bacterial presence. The Mantel-Haenszel method

was applied for the calculation of odds ratios (OR) and their respective 95% confidence interval (CI). A p value of ≤0.05 was considered as the criterion for statistical significance for these analyses.

Results

The study cohort consisted of 120 women with negative cervical cytology who served as controls and 62 cases of women with cervical cytology findings. Data concerning age-related characteristics in both groups of women are presented in Table 1. The women in the control group (mean age 29.8 years) were significantly younger that the women with cytology findings of the target group (mean age 40.7 years).

Mh and *Uu* were detected in both study groups and the prevalence rates are shown in Table 1. Presence of *Mh* cervical infection was confirmed in only one specimen (0.8%) from the control group, compared to 9 specimens (14.5%) originating from women with abnormal cytology. Specifically, the prevalence rates of *Mh* infection differed statistically significantly between the two groups and the probability for *Mh* presence was 20.21 times higher among women with any cytology finding (p<0.001, OR: 20.21, 95% CI: 3.12-125.72). A similar pattern was also observed for *Uu* infection: only 23 out of 120 women (19.2%) who served as controls were found positive for *Uu* infection, compared to 27 out of 62 women (43.5%) with cervical cytology findings. Statistical analysis revealed that the preva-

Table 1. Comparison between women with normal cytology (Controls) and those with any cytology finding (Cases), concerning mean age and frequency distribution of Mh and/or Uu presence

	Women with no cytology findings (Controls) n (%)	*Women with any cytology finding (Cases) n (%)*	*p*	*OR (95% CI)*
No. of specimens	120	62		
Age (years)			2.153*	
mean (± SD)	29.8 (± 7.2)	40.7 (± 13.4)		
median	29.0	40.0		
range	14 – 55	19 – 79		
Mh			<0.001**	20.21 (3.12 - 125.72)
yes	1 (0.8)	9 (14.5)		
no	119 (99.2)	53 (85.5)		
Uu			0.001**	3.25 (1.66 - 6.38)
yes	23 (19.2)	27 (43.5)		
no	97 (80.8)	35 (56.5)		
Mh + Uu			0.001**	17.6 (2.76 – 110.64)
yes	1 (0.8)	8 (12.9)		
no	119 (99.2)	54 (87.1)		

*Student's t-test. ** Two tailed Fisher's exact test.

Table 2. Women with cytology findings grouped according to the histological diagnosis and HPV, *Mh* and *Uu* infection status

Women with cytology findings (n = 62)									
	Infection status								
Histologic diagnosis	*HPV*	*HPV + Mh*	*HPV + Uu*	*HPV + Mh + Uu*	*Mh + Uu*	*Mh*	*Uu*	*none*	*Total (%)*
negative	1	0	0	0	0	0	0	0	1 (1.6)
cervicitis	1	0	0	0	0	0	0	2	3 (4.8)
condyloma	9	0	6	2	2	0	1	2	22 (35.5)
CIN 1	5	0	0	0	1	0	1	0	7 (11.3)
CIN 2	0	0	1	0	0	0	0	0	1 (1.6)
CIN 3	6	0	5	0	0	0	0	0	11 (17.8)
invasive cancer	7	1	5	3	0	0	0	1	17 (27.4)
Total (%)	29 (46.8)	1 (1.6)	17 (27.4)	5 (8.1)	3 (4.8)	0 (0.0)	2 (3.2)	5 (8.1)	62 (100)

lence rate of *Uu* in the latter group of women was 3.25 times higher (p=0.001, OR: 3.25, 95% CI: 1.66-6.38). The prevalence rates of *Mh* and *Uu* co-infection differed also statistically significantly between cases and controls. More specifically, women with cytology findings were shown to have a 17.6 times higher risk for presenting with *Mh* and *Uu* coinfection (p=0.001, OR: 17.6, 95% CI: 2.76-110.64).

The data concerning the prevalence rates of *Mh*, *Uu* and HPV cervical infection among women with cytology findings are shown in Table 2, segregated by the definite histological diagnosis of cervical specimens. The vast majority [22 out of 62 (35.5%) women] presented with the histological diagnosis of condyloma, followed by 19 cases (30.7%) who had a CIN diagnosis (either 1, 2 or 3). 17 women (27.5%) were diagnosed with invasive cervical cancer, 3 (4.8%) with cervicitis and only in one case (1.6%) the histology report was negative. HPV types were present in 52/62 (83.9%) of women with abnormal cytology: in 17 out of 22 (77.3%) women with the diagnosis of condyloma, in 17 out of 19 (89.5%) women with CIN 1,2 or 3 and in 16 out of 17 women (94.1%) with the histological diagnosis of invasive cancer. HPV prevalence was clearly increased in accordance with higher grades of cervical lesions. High –risk HPV types were found in 33 (53.2%) of women, and among them 28 (84.8%) were diagnosed with CIN or invasive cancer. Low-risk HPV types (LR-HPV types) were found in 19 (30.6%) women. *Mh* presence was confirmed in 4 women with condyloma (18.2%), in one woman with CIN (5.3%) and in 4 women (23.5%) with invasive cancer. *Uu* was present in half of the total number of specimens from women with the histologic diagnosis of condyloma (n=11, 50.0%), in 8 specimens (42.1%) with the histologic diagnosis of CIN 1, 2 or 3 and almost half the number of specimens with the diagnosis of invasive cancer (n = 8, 47.1%).

Statistical comparisons concerning the infection status of cases according to histological diagnosis and controls were also performed. Women with the histological report of condyloma had a 26.44 times higher risk for presenting with *Mh* infection, when compared with women in the control group (p=0.002, OR: 26.44, 95% CI: 3.68-183.94). The risk was 28 times higher for women with invasive cancer (p=0.002, OR: 28.00, 95% CI: 3.88-195.22), while it was not found to be statistically significantly different for women with CIN 1, 2 or 3 (p=0.256, OR: 6.61, 95% CI: 0.66-66.14). Concerning the *Uu* infection risk, this was 4.22 times higher among women with the histological report of condyloma compared to controls (p=0.005, OR: 4.22, 95% CI: 1.66-10.75), 3.07 times higher for women with CIN 1, 2 or 3 (p=0.037, OR: 3.07, 95% CI: 1.14-8.31) and 3.75 times higher for women with invasive cancer (p=0.025, OR: 3.75, 95% CI: 1.34-10.51). *Mh* and *Uu* co-infection risk was elevated among women with condyloma diagnosis and it was estimated to be 26.44 times higher compared to controls (p=0.002, OR: 26.44, 95% CI: 3.68-183.94). *Mh* and *Uu* co-infection risk was also elevated for the group of women with invasive cervical cancer (p=0.006, OR: 25.50, 95% CI: 3.34-188.18) but it was not significantly different for CIN 1, 2 or 3 women (p=0.256, OR: 6.61, 95% CI: 0.66-66.14) compared to women in the control group.

Concerning HPV presence and the diagnosis for cancer among the group of cases, any type HPV DNA presence was associated with invasive cancer diagnosis from cytology (p<0.001, OR: 69.00, 95% CI (9.04-482.94), while only HR-HPV DNA presence was associated with the same diagnosis from histology (p=0.001, OR: 11.25, 95% CI: 2.51-48.93). There was no case of either *Mh* or *Uu* infection, or *Mh* and *Uu* co-infection associated with the HPV

Table 3. Comparison of HPVs prevalence among women with cytology findings according to *Mh* and *Uu* infection status

	Women with cytology findings (n = 62)			
	HPV + N n (%)	*HPV- n (%)*	*p*	*OR (95% CI)*
Mh			0.151	0.304 (0.066 - 1.360)
yes	6 (11.5)	3 (30.0)		
no	46 (88.5)	7 (70.0)		
Uu			0.735	0.733 (0.200 - 2.683)
yes	22 (42.3)	5 (50.0)		
no	30 (57.7)	5 (50.0)		
Mh + Uu			0.111	0.248 (0.052 - 1.148)
yes	5 (9.6)	3 (30.0)		
no	47 (90.4)	7 (70.0)		

infection status in women with cytology findings, as depicted in Table 3. Even when the cervical specimens were grouped according to the histological report (negative, cervicitis, condyloma, CIN 1-2-3, invasive cancer), no such association emerged within the groups.

Discussion

HPV is considered as the main aetiological factor in the development of cervical cancer, but other factors, such as concomitant infections of the genital area, have also been investigated as potentially involved in the development of cervical cancer. Despite the fact that the effects of *Uu* and *Mh* on the cervical epithelium remain unclear to some extent, there is accumulating evidence that HPV affected cervical epithelium may become more susceptible to *Uu* and *Mh* infections or epithelial cells infected with *Uu* and *Mh* may become more susceptible to infection with high-risk HPV types leading to HSIL and cervical cancer.

In the present study, we investigated the presence of *Uu* and *Mh* alone and in coexistence with HPV infection in women with abnormal and normal cervical cytology. A high prevalence rate of *Uu* was found in women with abnormal cytology (43.5%), compared to the control group (women with no cytology findings) (19.2%). *Uu* was detected in the majority of women with abnormal cytology: in half cases (47.1%) of invasive cancer, and in 42.1% of patients with histological diagnosis of CIN 1, 2 and 3 lesions. The role of Ureaplasmas as a possible cofactor in HPV infections is rarely addressed in the literature. Our results are in accordance with other microbiological studies, which reported a high association between *Uu* infection and the grade of cytological cervical lesions (a 35% rate of *Uu* in low-grade squamous intra-epithelial lesions, L-SIL and 45% in high-grade intra-epithelial lesions, H-SIL), while only 19% of the control group samples were positive for *Uu* [22]. Furthermore, according to another study which was conducted to estimate the prevalence of sexually transmitted infections associated with HPV, from patients who underwent annual routine gynaecological exams, the most frequent infection was *Ureaplasma* species [23]. Similarly, a recent systematic meta-analysis reported a high incidence of *Uu* in women with LSIL and HSIL lesions, suggesting that *Uu* infection may be a contributing factor in the progression of genital lesions leading to abnormal cervical cytopathology [12,15].

Interestingly, the co-infection of HPV and *Uu* was higher in patients with CIN 3 and invasive cancer. This finding is very close to Lukic et al study who reported an interesting association of HPV and *Uu* in patients with HSIL vaginal cytology (83%) [21] while two more studies have found a significant increase of *Uu* in women with cervical cancer compared to control group [24].

In this study the risk for *Mh* infection was estimated to be 20.21 times higher among women with any cytology findings ($p<0.001$) compared to the control group and is in agreement with other studies [12,15,23]. Women with invasive cancer had 28 times higher risk for presenting *Mh* infection compared to the control group. *Mh* and *Uu* co infection was statistically significant between cases and controls: women with cytological findings were shown to have 17.6 times higher risk for having *Mh* and *Uu* co-infection ($p=0.001$).

Although in the present study neither *Mh* nor *Uu* co-infection were significantly associated with the HPV infection status in women with cytology findings, a few other studies observed a significant association of *Uu* presence in HPV positive women [25]. In contrast, other investigators although have reported a low prevalence of concurrent HPV and *Uu* infection and consequently lack of association between *Uu* infection and HPV in the cervical lesions, the HPV and *Ct* co-infection was found significant [26,27]. Interestingly, specific polymorphisms in critical immuno-motivative molecules, such as interleukins IL-4R/IL-10, have been detected in HPV-related cervical intraepithelial neoplasia [28]. Moreover, another study conducted in sexually active women without cervical lesions, presented no significant association between HPV, *Mh* and *Uu* [29,30].

Finally, according to the findings of the present study, it can be concluded that the presence of *Uu* in CIN 3 and invasive cancer lesions, may be implicated to manifestations of the cervical epithelial cells. In addition, the persistent and recurrent inflammation of HPV, and the infection with *Uu* may enhance inflammatory responses related to the progression of lesions in the female genital tract increasing the risk for abnormal cytopathology and cervical neoplasia. Nevertheless, and since the results of the present study and the limited data presented by other scientists remain contradictory, further investigations are required, as the exact activity and the synergistic role of these infectious agents is not clarified, thus their role in the progression of cervical dysplasia cannot be excluded. Long-term larger population studies are required to enlight the effects of these frequent coinfections in the female genital track. Therefore, it seems that screening for simultaneous genital infections especially in CIN-HPV positive women may prove extremely important

for the early and rapid treatment of patients aiming to decrease the probable cumulative risk of infection persistence and progression of lesions on cervical epithelium.

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Conflict of interests

The authors declare no conflict of interests.

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