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Causative relations between human papilloma virus infection and cervical intraepithelial neoplasia

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ABSTRACT

The aim of this study was to assess the correlation between human papilloma virus (HPV) infections and cervical intraepithelial neoplasia (CIN). The study included 421 women (aged 18–45 years) who were examined gynecologically; their medical history and Pap smear results were collected and colposcopy and HPV tests were performed. In those cases where colposcopy and cytological evidence of atypism was found, biopsy or abrasion from the uterine cervix was performed. The GenoFlow HPV Array Test Kit was used to analyse the HPV status in cervical samples collected during the study. The obtained results showed that, one/more HPV genotypes were identified in 42% (177/421) and HPV(–) in 58% (244/421) of the examined women. In the HPV(+) group, CIN was diagnosed in 57 (13.5%) women, whereas, in the HPV(–) group, in 44 (10.5%) women. There was a significant dependence between HPV(+) status and development of CIN ($p = 0.001$), but the statistical analysis did not reveal sufficient positive predictive value or precision ($p > 0.05$), i.e. probability that (randomly selected) HPV(+) patients have CIN; neither sensitivity ($p > 0.05$), i.e. probability that (randomly selected) CIN patients are HPV(+); nor specificity ($p > 0.05$), i.e. probability that (randomly selected) women without CIN are HPV(–). These results indicate that infection with HPV is probably not the only cause for development of CIN.

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Introduction

The 2012 Consensus Guidelines published by the American Society for Colposcopy and Cervical Pathology (ASCCP) and its partner organizations utilize a two-tiered system in which histologically diagnosed cervical intraepithelial neoplasia (CIN) which covers 1/3 of epithelial layers (CIN I) is classified as a low-grade squamous intraepithelial lesion (LSIL) and CIN that covers more than half of the epithelial layers of the uterine cervix (CIN II/III), as high-grade precursor (HSIL).[1] Following the histologic identification of CIN, treatment is recommended for high-grade pre-invasive lesions.[1]

Human papilloma virus (HPV) is recognized as a main etiological agent causing CIN.[2] The major factor in cervical carcinogenesis is HPV infection, with HPV persistence for a certain period of time, progression to precancer and invasion.[2] To date, more than 150 HPV genotypes have been characterized, and about 40 of them infect the genital tract.[2] HPV types are commonly classified as low-risk or high-risk types according to their role in the development of cervical cancer.

HPV infection alone may not be sufficient to cause cervical lesions and cancer.[3] There is evidence that

most HPV infections are transient and clear spontaneously within 12–24 months after the first detection.[3] Persistent HPV infection with elevated risk of cervical cancer occurs in only a small percentage of the virus-infected women.[3]

The aim of this study was to assess the correlation between HPV infections and CIN.

Subjects and methods

Study cohort

The study was carried out retrospectively using the results database of the Genetic Medico-Diagnostic Laboratory "Genica", Sofia, Bulgaria, and the Clinic of General and Oncogynecology at the Military Medical Academy (MMA), Sofia, Bulgaria. The results from a three-year period (2011–2014) were studied. We included 421 women in the study, aged 18–45 years, who voluntarily attended a gynecological clinic for HPV screening (to determine the presence or absence of infection) or for a routine gynecological check-up. Their medical history and Pap smear results were collected, and colposcopy and HPV tests were performed for each patient.

All patients included in the study gave their informed consent and the study was approved by the MMA ethical committee. All data collected and used in the study concerning the patients' personal details, age, previous treatment for HPV infection, including surgical or destructive treatment of the cervix, co-morbidities, etc. were treated in compliance with the data protection rules (Anonymity Clause).

Exclusion criteria

The following groups of women were not included in the study: pregnant, diagnosed with immune diseases, previously diagnosed with HPV infection, history of previous surgical or destructive treatment of the cervix, precancerous conditions and previous HPV vaccine.

Diagnosis and sample collection

Diagnostic colposcopy

Diagnostic colposcopy was performed at the Clinic of General and Oncogynecology, MMA, using an Olympus colposcope (OCS5 – ZB). In standard colposcopy examination, the surface of the cervix and vaginal vaults were cleaned from mucus and secretions with saline lavage and examined. Then, coating with 3%–5% solution of acetic acid was applied under normal lighting conditions and through a green filter, finally followed by coating with Lugol solution (Schiller's test). Punch biopsy of changes in the cervix was taken and the samples were forwarded to the Department of Pathology for further processing (CIN+/-).

Pap test

The Pap test was taken from the front and rear lip and the cervical canal. From each sample taken, a cytological smear was made, which was fixed on a microscope slide and stained by the method of Papanicolaou.[4] After the staining was performed, the cellular morphological characteristics were analysed by light microscopy (Binocular microscope OPTIKA – B380) to detect signs of cervical benign and malignant cells or other pathological changes. In the group where Pap III or higher grade was found, we conducted curettage from the uterus cervix and the samples were forwarded to the Department of Pathology for further analysis (CIN+/-).

HPV typing

HPV typing (GenoFlow HPV Array Test Kit) was performed to detect HPV infection, especially high-risk HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68; moderate-risk genotypes: 53, 73, 81 and 82; and low-risk genotypes: 6, 11, 42, 43, 44, 40, 61, 54, 55, 70, 57, 71, 72, 84 and 26. Cervical swabs were taken and

analysed at the Genetic Medico-Diagnostic Laboratory "Genica" LTD, Sofia, Bulgaria.

Sample storage

Cervical cell samples were stored in liquid transport medium (PreservCyt solution, Hologic Corporation). Specimens were stored at 5–30 °C during transportation and were kept at room temperature up to two weeks.

DNA extraction

Approximately 1–2 mL of sample was used for each test. The samples were centrifuged for 5 min and the pellets were resuspended in 200 µL phosphate buffered saline buffer. DNA extraction was done using a QIAGEN QIAamp Blood Mini Kit. Eluted DNA in QIAGEN Buffer AE (50–200 µL) was stored at –15 to –20 °C until further analysis.

Amplification of target DNA

GenoFlow HPV Array Test Kit (FT-PRO, GF assay; DiagCor Bioscience Inc., Hong Kong) was used. Polymerase chain reaction (PCR) was performed in a final volume of 25 µL and the thermal profile suitable for GeneAmp PCR System 9700 (Applied Biosystems) was as follows: 95 °C for 9 min; 43 cycles of 95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s; and finally 72 °C for 5 min. To monitor the test performance we used DNase-free water (negative control) and HPV DNA control (positive control; non-infectious DNA containing an HPV sequence).

Flow-through™ hybridization

GenoFlow HPV Array Test Kit (FT-PRO) was used in an FT-PRO Flow-through™ System Set-up. After denaturation of the PCR products at 95 °C for 5 min, they were immediately placed into an ice bath for at least 2 min. Hybridization and colorimetric detection of the samples were done according the manufacturer's protocol.

Data interpretation

Interpretation of the results was done using data analysis strips and an image capture system for scanning, analysis and storage of the membrane image.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics. The dependence between the variables was assessed by the Pearson correlation coefficient and Matthews correlation coefficient, also known as the *phi* coefficient. *Chi*-square test was used to determine whether there is a significant difference between the expected frequencies and the observed frequencies. The following statistical measures of the performance of a binary

classification test were taken into account: positive predictive value (PPV) or precision (probability that a randomly selected patient diagnosed with a condition has the condition); sensitivity (probability that a randomly selected patient with a condition is diagnosed with the condition); specificity (probability that randomly selected healthy people are diagnosed as healthy). The results were considered statistically significant at significance level $\alpha < 5\%$.

Results and discussion

The results presented in Table 1 show that, among all the 421 women included in the study, one or more HPV genotypes were identified in 177 women (42%) and HPV(–) results were obtained for 244 (58%) women. In the HPV (+) group, based on the histopathological results, 57 (13.5%) women were shown to have CIN(+) results, whereas in the HPV(–) group, the incidence was 10.5% (44 women).

There was a statistically significant difference ($p < 0.05$) between HPV(+) results and development of CIN (Table 2). However, the statistical analysis did not reveal a PPV or precision ($p \geq 0.05$), i.e. probability that (randomly selected) HPV(+) subjects have CIN; or sensitivity ($p \geq 0.05$), i.e. probability that (randomly selected) CIN patients are HPV(+); or specificity ($p \geq 0.05$), i.e. probability that (randomly selected) negative CIN is HPV(–). The Pearson correlation analysis for linear dependence between two variables (HPV infection and CIN) showed low correlation ($r = 0.122$), indicating disagreement between prediction and observation. Pearson correlation coefficient for HPV infection and CIN in our study coincides with the *phi* coefficient (also known as the mean square contingency coefficient, or Matthews' correlation coefficient). In our study, the *phi* coefficient ($\varphi = 0.122$) for HPV(+) and CIN also indicated disagreement between prediction and observation.

It is generally considered that there is a close relationship between high-risk HPV genotypes infection and cervical cancer development. Although HPV 16 and 18 were acknowledged as a central and independent cause of most cases of cervical cancer based on molecular analysis in 2002,[5] the incidence of HPV 16 and 18 infections has not been proved to clearly correlate with the

Table 2. Chi-square test for HPV(+/-) and CIN(+/-) patients.

	Value	df	Asymp. sig. (2-tailed)	P (2-tailed)	P (1-tailed)
χ^2	11.297 ^a	1	0.001		
Continuity correction ^b	10.533	1	0.001		
Fisher's exact test				0.001	0.001
Number of valid cases	421				

Note: df, degrees of freedom.

^a0 cells (0.0%) have expected count less than 5. The minimum expected count is 42.46.

^bComputed only for a 2×2 table.

incidence and mortality of cervical cancer worldwide.[6] As reviewed by Wilyman,[6] since a considerable part of the HPV 16/18 infections has not been reported to lead to cervical cancer, this indicates that other etiological or 'risk' factors are necessary for persistent HPV infection to progress to cancer. Moreover, epidemiological studies have reported that HPV is a very common sexually transmitted virus, with prevalence between 10% and 40% in women with no cytological abnormalities, suggesting that a large part of HPV infections do not ultimately cause CIN (for review see [1]). Likewise, since acquired immunity to HPV may develop in women at a more advanced age, a large part of the population may be expected to have had a subclinical HPV infection at some point in their lives, particularly at a younger age.[1] Thus, while it is well known that HPV infection is implicated in the process of cervical carcinogenesis, other risk factors which increase the likelihood of HPV infection, such as high alcohol consumption and more sexual partners, are also likely to be involved in the development of a malignant phenotype.[1]

Although many studies find a close relation between HPV infection and development of CIN, they also show that there are some women who have CIN but not HPV infection.[7–10] For example, a recent study conducted by the Northern Ireland HPV Working Group aimed to demonstrate the HPV genotypes predominating in pre-malignant and cervical cancers in Northern Ireland before the vaccination campaign has effect.[7] The authors reported an HPV type-specific prevalence of 48.1%, 65.9%, 81.3%, 92.2% and 64.3% among CIN grades I–III, squamous cell carcinomas (SCC) and adenocarcinoma (AC) cases, respectively. There was only one HPV genotype detected in over 80% of SCC cases and an HPV(–) result in almost a third (32%) of all cervical pathologies, including 51.9% of CIN I, 34.1% CIN II, 18.7% of CIN III, 7.8% of SCC and 35.7% of AC cases.[7] Haghshenas et al.[8] tested a total of 98 paraffin-embedded cervical samples consisting of 63 SCCs, 4 ACs, 19 CIN I, 4 CIN II and 8 CIN III cases diagnosed in 2009–2011, with high-risk HPV genotyping DNA test. Of the 98 cervical samples analysed by PCR, 78 (79.59%) were positive for HPV DNA and 20.41%, negative. HPV was detected in 52

Table 1. Results for HPV(+/-) and CIN(+/-) patients.

		HPV infection				Total	
		Negative		Positive		n	%
		n	%	n	%		
CIN	Negative	200	47.5	120	28.5	320	76
	Positive	44	10.5	57	13.5	101	24
	Total	244	58	177	42	421	100

of SCC, 4 of AC, 14 of CIN I, 4 of CIN II and 4 of CIN III cases.[8] In comparison, the percentage of women with CIN who were negative for HPV (10.5%) was not as high in our study. As reviewed by Reboli et al.,[9] the ability of HPV testing to detect CIN is associated with an increased frequency of positive tests without underlying CIN, which increases the need for colposcopy and repeated testing. Mongelós et al.[10] studied the frequency of high-risk HPV by HPV DNA test according to the cytology results in 122 women treated for squamous intraepithelial lesions of the cervix. Of 79 women (75%) treated for LSIL and 43 (35%) ones, for HSIL, only 28% (34/122) were positive for high-risk HPV, and 72% (88/122) were negative.[10]

Taken together, the results from our study are in agreement with other reports [7–10] that there is not always a clear-cut causative relation between HPV infection and CIN. Although HPV persistence is widely recognized to play an essential role in the progression of pre-neoplastic lesions and the development of cervical cancer, the increasingly growing body of evidence seems to suggest that HPV is probably not the sole reason for the development of CIN. So there is a need for further extensive research to identify other potential risk factors implicated in CIN in addition to HPV for future prevention strategies.

Conclusions

The results from this study showed that, although a significant dependence was observed between HPV(+) status and development of CIN in the studied cohort, the HPV(+) status may not be considered a specific and sensitive predictive factor for the development of CIN. Our results are part of a growing amount of data from many clinical investigations all suggesting that HPV could not likely be postulated as the sole causative agent in all CIN cases. Further research is needed to establish all the reasons for the development of CIN and uterine cervix cancer, respectively.

Disclosure statement

No potential conflict of interest was reported by the authors.

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