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SINGLE AND MULTIPLE-TYPE OF HPV INFECTIONS IN WOMEN WITH CERVICAL CANCER AND PRECANCEROUS LESIONS IN THE SOUTHWESTERN REGION OF THE REPUBLIC OF NORTH MACEDONIA

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Abstract

Background: Cervical cancer is recognized as the third most prevalent type of cancer and it is the fourth cause of death in women worldwide. It is also recognized as a second more prevalent type of cancer in women of 44 years or younger. Currently, over 100 HPV types have been identified, but HPV16 and 18 are recognized as the mayor malefactors in cervical carcinogenesis. Individuals are often infected with multiple genotypes of human papillomavirus (HPV) simultaneously, but the role that these infections play in the development of cervical disease is not well established.

Aim: Our objective was to assess the relationships between single and multiple-type HPV infections with patients' age and histological status of cervical lesion. Material: In the period of two and a half years (March 1,2017 till September 30,2019) the total number of 212 women (18-65 years old) who were positively screened for opportunistic cervical cancer by pap smears, were sent by their family gynecologist to the Center for Public health of Bitola for HPV genotyping.Method: Samples of HPV genotyping were collected by scraping the epithelium out of the cervical canal and transferred into 1.5 ml plastic tubes and transport medium using a disposable sterile probe.. DNA extraction was conducted using the kit for isolation of nucleic acids, PureLink Genomic DNA kit, Invitrogen, produced by "Life-Technology". After the extraction of DNA from the specimens, the presence of 21 HR-HPV genotypes were detected by The DNA-Technology HPV Quantitative Real-time PCR Kit,DTlite, LLC, Russia ®.The distribution of the data is shown in absolute and relative numbers. Non-parametric Chi-square test was used to test for differences. The p value of <0.05 was considered statistically significant.Results: Among 212 women, 79 were diagnosed with non high risk cervical lesions (non-hrCL) and 133 were with high risk of cervical lesions (hrCL). The most dominant HPV genotypes were: 16 (19,0%), 31 (12,2%), 52 and 59 (7,1%), 33(6,8%) and 18 (6,5%). Patients with multiple-type HPV infections had a significantly higher prevalence than those with single type of HPV in the age groups of up to 24 years and from 25 to 34 years, 16.5% vs 11.8%, and 51.8% vs 33.1%, respectively. However, statistical analysis did not confirm a significant difference between women with single and multiple types of HPV infections in relation to the cytological finding.Conclusion: In this study of 212 women, HPV16 was most prevalent type, detected in 19% of all HPV positive cases and with the highest prevalence rank in ST groups with 30,5%. Other most prevalent types were HPV type 31, 52, 59, 33 and 18 with the presence of 12,1%; 7,1%; 7,1%; 6,8%, 6,5% respectively. Patients with multiple type HPV infections had a significantly higher prevalence than those with single type of HPV in the age groups of up to 34 years. A better understanding of the interactions among HPV genotypes is of great interest, not only for risk assessment of hrCLs in women with multiple HPV infections, but also for the development of the next generation of HPV vaccines by

predicting the possible emerging genotypes that compete with those targeted by current vaccines.

Keywords: Human papillomavirus, multiple HPV type infections, genotype, age

ИНФЕКЦИИ ПРЕДИЗВИКАНИ СО ЕДЕН ИЛИ ПОВЕЌЕ ТИПОВИ НА ХУМАН ПАПИЛОМА ВИРУС (ХПВ) КАЈ ЖЕНИ СО КАРЦИНОМ НА ГРЛОТО НА МАТКАТА И ПРЕКАНЦЕРОЗНИ ЛЕЗИИ ВО ЈУГОЗАПАДНИОТ РЕГИОН НА РЕПУБЛИКА СЕВЕРНА МАКЕДОНИЈА

Апстракт

Вовед: Ракот на грлото на матката е трет по застапеност карцином во светот, и се препознава како четврта причина за смрт поради карцином кај жените. Тоа е втор најчест вид на карцином кај жени од 44 години или помлади. Перзистентна инфекција со хуман папиломавирус (ХПВ) се смета за главен фактор кој предизвикува карцином на грлото на матката. Идентификувани се повеќе од 100 типови ХПВ, од кои 40 се сексуално преносливи. Кај пациентките честопати се детектира инфекција со повеќе генотипови на ХПВ истовремено, но улогата што ја играат овие инфекции во развојот на заболувањата на грлото на матката не е докажана.

Цел на трудот е да се проучат односите помеѓу инфекции предизвикани со еден или повеќе типови на ХПВво зависност од возраста на пациентките и цитолошкиот наод на цервикалната лезија.

Материјал: Во период од 2,5 години (1.03.2017 до 30.09.2019 година) вкупно 212 жени (18-65 години) беа вклучени во студијата. Тие беа упатени од матичните гинеколози во Центарот за јавно здравје Битола за ХПВ генотопизација, поради позитивен резултат на скрининг ПАП тестот за карцином на цервиксот на матката.Метод: Примероците за генотипизација на ХПВ беа собрани со стружење на епителот на цервикалниот канал и беа транспортирани во пластични епрувертки за еднократна употреба, со волумен од 1,5 ml. Екстракција на ДНК беше спроведена со помош на комплетот за изолација на нуклеинските киселини, комплет за геномска ДНК PureLink, Iivitrogen изработен од „Life-Technology". Присуството на ХПВ генотиповите беше детектирано со DNA-Technology HPV QUANT, QuantitativeReal-time PCR Kit, DTlite, LLC, Russia. Кит наменет за специфична идентиификација, типизација и квантификација на нискоризични (HPV 6, 11, 44) и високоризични (HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) ХПВ-генотипови во однос на нивниот онкоген потенцијал. Дистрибуцијата на податоците е прикажана со апсолутни и релативни броеви. За тестирањето на разликите беше користен непараметарски Chi-square test. За статистички значајна земена е вредноста на $p<0,05$.Резултати: Кај 212 жени, 133 биле дијагностицирани со висок ризик на цервикална лезии и 79 со низок ризик на цервикална лезии. Преваленцата на ХПВ инфекции предизвикани со различни ХПВ генотипови покажа еден врв на возраст кај жени помеѓу 25 и 34 години, кој опаѓа со возраста. Најчесто изолирани ХПВ генотипови беа ХПВ 16 (19,0%), 31 (12,2%), 52 и 59 (7,1%), 33 (6,8%) и 18 (6,5%). Инфекција со повеќе типови на ХПВ беше статистички значајно почеста од ХПВ инфекција со еден тип, кај пациентките во возрасните групи до 24 години и од 25-34 години, 16.5% vs 11.8% и 51.8% vs 33.1%, консеквентно, но статистичката анализа не потврди сигнификантна разлика меѓу инфекции со еден или повеќе типови во однос на цитолошкиот наод.

Заклучок: Во оваа студија, ХПВ16 беше најчесто изолиран тип, детектиран кај 19% од сите ХПВ позитивни случаи. Другите најзастапени типови беа ХПВ тип 31, 52, 59, 33 и 18 со 12,1%; 7,1%; 7,1%; 6,8%, 6,5%, соодветно. Пациентите со ХПВ инфекции со повеќе типови беа статистичли значајно повеќе застапени од оние со единечен ХПВ тип кај жени до 34 години. Подобро разбирање на интеракциите помеѓу генотиповите на ХПВ е од голем интерес, не само за проценка на ризикот од појава на цервикален карцином кај жени со инфекции со повеќе типови на ХПВ, туку и за развој на следната генерација на вакцини против ХПВ, со што би се предвиделе новите можни генотипови кои најчесто се детектираат и кои делуваат синергистички со оние кои се вклучени во тековните вакцини.

Клучни зборови: Хуман папиломавирус, инфекција со повеќе типови на ХПВ, генотип, возраст

Introduction

Cervical cancer is the third most prevalent cancer worldwide, being recognized as the fourth cause of death due to cancer in women. It is the second more prevalent type of cancer in women of 44 or younger (1). Human papillomavirus (HPV) persistent infection is considered the causal factor for pre invasive and invasive cervical carcinoma. More than 100 HPV types have been identified and 40 are sexually transmitted. These are further categorized into two groups: low risk HPV types (lr-HPV) and high-risk HPV types (hr-HPV), depending on their relative risk of causing human malignancy. High-grade intraepithelial lesion and invasive carcinoma, are mostly associated with oncogenic HPV types included in alpha-9 (HPV 16, 31, 33, 35, 52, 58 and 67) and alpha-7 (HPV18, 39, 45, 59, 68 and 70) species, considering that the types belonging to a species have 80% of genetic similarity (2). Among these types, HPV 16 and 18 infections, followed by HPV 31 and 45 are found in more than 80% of cervical cancer specimens (3, 4, 5, 6).

Several studies have demonstrated that multiple-type of HPV infections are a significant risk factor for high-risk cervical lesions (hrCLs) (7,8,9). Conversely, alternate studies showed that multiple- type HPV infections (MT-HPV infections) have no synergistic or additive effect on the development of cervical diseases or increased risk for hrCLas compared to a single-type of HPV infection (ST-HPV infections) (10,11). However, there is no consensus whether MT-HPV infections are associated with higher risk of carcinogenesis than ST-HPV infections and it is not also clear that in women with multiple-type of HPV infections whether a competitive or cooperative interaction exists among the co-infecting genotypes (10,12,13).

Aim

In this study, the prevalence of ST and MT-HPV infections in women with squamous and glandular lesions of different age strata has been analyzed. The objectives were to evaluate whether ST and MT differ 1) in its association with the type of the cervical lesion and 2) according to the patients age.

Material and Methods

Study Design and Population

A total number of 212 women were enrolled in this cross- sectional study, which took place from March 1,2017 till September 30,2019 in the Center for Public Health in Bitola.

All 212 women have firstly attended a primary screening program and were referred to the Center for Public Health in Bitola for HPV DNA genotyping from general gynecologists, after getting an abnormal pap smear.

The Criteria for women included in the study were: to be between 18 and 64 years of age, to have at least one identified HPV type on HPV DNA testing and to have positive data on the result of the Pap test. The exclusion criteria for women in this study were: prior hysterectomy, virgins and pregnancy.

Cytology Classification

Conventional Pap tests were performed for cytological evaluation, and the findings were interpreted according to the criteria set by the 2004 Bethesda System for cervicovaginal cytology reporting. The cases were divided into two general groups based on cytological diagnoses such as: 1) those with hrCLs, including atypical squamous cells, which cannot exclude high-grade squamous intraepithelial lesion (ASC-H) and low grade squamous intraepithelial lesion with atypical cells, which cannot exclude high-grade squamous intraepithelial lesion (LSIL-H) with high-grade squamous intraepithelial lesion (HSIL), and 2) those with non-hrCL, including atypical squamous cells of undetermined significance (ASC-US) and low-grade squamous intraepithelial lesion (LSIL), and negative for intraepithelial lesion or malignancy (NILM).

HPV DNA Extraction and Detection

DNA samples were collected by scraping the epithelium of the cervical canal and were transferred into 1.5 ml plastic tubes and transported using a disposable sterile probe. Storage and transportation of the material was carried out according to current regulations. DNA extraction was conducted using a kit for isolating nucleic acids, PureLink Genomic DNA kit, INVITROGEN developed by "Life-Technology". The kit is based on the selective binding of DNA to silica- based membrane in the presence of chaotropic salts. The cells are digested with Proteinase K at 55°C using optimized digestion buffer formulation that aids in protein denaturation and enhances Proteinase K activity. Any residual RNA is removed by digestion with RNase A prior to binding samples to the silica membrane. The lysate is mixed with ethanol and binding buffer that allows high DNA binding on Spin Columns. The DNA binds to the silica based membrane in the column and impurities are removed by thorough washing with Wash Buffers. The genomic DNA is then eluted in low salt Elution Buffer.

Genotyping for 21 HPV types was conducted using real-time PCR detection with a reagent kit developed by "DNA-Technology" (HPV Quant-21, Russia). This test detects 18 high risk-HPV types (HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 16 low-risk-HPV types (HPV 6, 11, 44). The kit contains 8-tube- strips, paraffin sealed PCR–mix, which contains target-specific hydrolyzing probes. Real-time PCR technology is based on measurement of fluorescence at every cycle of reaction. The intensity of fluorescence is analyzed with a Real-time PCR instrument data collection unit and the software provided.

Only cases that had satisfactory β-globin levels were included in the study. A HPV infection was classified as a single-type and multiple-type infections. Multiple-type infections include the association with hr and/or lr-HPV types.

The distribution of the data is shown in absolute and relative numbers. Non-parametric Chi-square test was used to test for differences. The p value of <0.05 was considered statistically significant.

Results

The single-type and multiple- type of HPV infections in the screening population were stratified by age. Out of a total number of 212 women, 131 (61,79%) had single-type HPV infection, while 81 (38,21%) women had multiple-type HPV infection (Fig. 1).

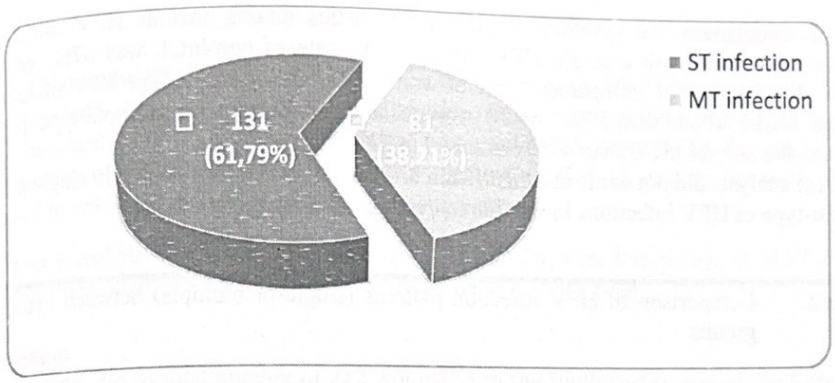

Figure 1 Number of women with single-type HPV infection (ST-HPV infection) and women with multiple-type HPV infection (MT-HPV infection)

From a total number of 212 women who were included in the study: 29 or 13.7 % were 25 years old or younger, 88 or 40.6% were between 25 and 34 years old, 62 or 28.3% were between 35 and 44 year olds and 34 or 15.1% were older than 45 years.

The highest frequency of HPV DNA positive samples of the screening population was in the age group of 25-34 years old (40.6% of tested women) and gradually decreased with increasing age (Table 1).

Patients with single and multiple-type HPV infection were significantly different by age stratification ($p < 0.05$). In younger patients, in the age group of women from 18 to 24 years old and in the age group from 25 to 34 years of age multiple-type HPV infections had a significantly higher prevalence than those with single-type HPV infections (16.5% vs 11,8% and 51,8% vs 33,1% consecutively). At the strata age of 35-44 year olds, the prevalence of ST- HPV infections and MT-HPV infections swaps in favour of single- type of HPV infections (32,3 % vs 22,4% and 19,7% vs 8,2%) but statistical analysis did not confirm a significant difference (Table 1).

Table 1 Age stratified proportion of single type (ST) and multiple-type (MT) HPV infections

Age of women	Total		ST infection		MT Infection	
	N	%	N	%	N	%
<24	29	13.7%	15	11.8%	14	16.5%
25 - 34	88	40.6%	47	33.1%	41	51.8%
35 - 44	62	28.3%	43	32.3%	19	22.4%
45 - 54	27	12.3%	23	17.3%	4	4.7%
55 - 64	6	2.8%	3	2.4%	3	3.5%
Total	212	100.0%	131	100.0%	81	100.0%

Chi-square = 12 p=0.018 sig

Single and multiple-type HPV infections, stratified by cytology, could be seen in table 2. In both groups of women: 133 with hrCL and 79 with non- hrCL, a single genotype infection of hrHPV was predominant.

Table 2 summarizes the cytology results and statistics among various HPV infection patterns. In women with a single HPV infection, the rate of non-hrCL was 67% or 53 women, and was higher compared to rate of women infected by single-type HPV infection and had hrCL, which was 59% or 78 women and in women with multiple-type HPV infection, the rate for hrCL was 41% compared to 33% for non-hrCL.
Statistical analysis did not confirm a significant difference between women with single and multiple-type of HPV infections in relation to cytological findings (p = 0.22).

Table 2 Comparison of HPV infection patterns (single or multiple) between cytology groups

	1 HPV type		2 HPV types		3 HPV types		4+ HPV types		total	
cytology group	N	%	N	%	N	%	N	%	N	%
Non-hrCL	53	67.0%	15	19.0%	7	8.9%	4	5.1%	79	100.0%
hrCL	78	59.0%	31	23.1%	19	14.2%	5	3.7%	133	100.0%

Chi-square = 1.50 p=0.22

Table 1 HPV types in single and multiple-type of HPV infections

	total		ST- HPV infection		MT- HPV infection	
HPV type	N	%	N	%	N	%
6	4	1.2%	0	0.0%	4	1.9%
16	65	19.9%	40	30.5%	25	11.8%
18	22	6.7%	9	6.9%	13	6.1%
26	1	0.3%	1	0.8%	0	0.0%
31	31	9.5%	12	9.2%	29	13.7%
33	23	7.0%	9	6.9%	14	6.6%
35	6	1.8%	0	0.0%	6	2.8%
39	12	3.7%	6	4.6%	6	2.8%
44	4	1.2%	0	0.0%	4	1.9%
45	10	3.1%	4	3.1%	6	2.8%
51	20	6.1%	7	5.3%	14	6.6%
52	24	7.3%	8	6.1%	16	7.5%
53	11	3.4%	3	2.3%	8	3.8%
56	11	3.4%	4	3.1%	7	3.3%
58	14	4.3%	6	4.6%	8	3.8%
59	24	7.3%	5	3.8%	19	9.0%
66	19	5.8%	7	5.3%	12	5.7%
68	19	5.8%	8	6.1%	11	5.2%
73	6	1.8%	1	0.8%	5	2.4%
82	1	0.3%	1	0.8%	0	0.0%
total	327	100.0%	131	100.0%	212	100.0%

Table 3 shows the HPV types in ST- and MT- HPV infections. HPV16 was most prevalent type, detected in 65 cases (19% of all HPV positive cases) and topped the prevalence rank in ST groups with 30,5%. Other most prevalent types were HPV type 31, 52, 59 and 33 with the presence of 12,1%; 7,1%; 7,1%; 6,8%, respectively. HPV 18 was on the sixth place, detected in only 22 cases (6.5% of all HPV positive cases).

All other tested HPV types, except of types 16, 18 and 26, were less prevalent in ST- HPV infections than their MT counterparts containing the same HPV type.

Discussion

In this study, out of total number of 212 women from the southwest region of the Republic of North Macedonia with pre-invasive and invasive cervical lesions, HPV16 was detected in 65 or 19% of the women. The prevalence of HPV16 was followed by other HPV types, HPV 31, 52, 59 and 33 and HPV18 appeared in the sixth position, as in other studies (3,4,5,6,8).The frequency of multiple-type HPV infections reached their maximum in the age range from 25-34 year olds and gradually decreased thereafter. This findings are in accordance with other studies, as that of Salazar et al. (10), but Resende et al.(3) in his study found a bimodal prevalence curve for MT-HPV infections with prevalence peaks at a very young (<29 years) age and in women between 50–59 years of age. Several published studies have shown that the risk for hrCL decreased with increasing number of co-infecting genotypes, especially when the infections involved 3+ hrHPV genotypes, with explanation that the weak and ineffective immunity generated by each HPV genotype may collectively provide an overall stronger immunity against HPV infection through antibody cross-reactions (10,11,13). However, other authors found a significant association between MT-HPV infections and disease severity and concluded that the infection with multiple types of HPV is a significant risk factor for high-grade CIN (3,8,9,16). A consistent claim with this study was in spite of the limited sample size, and, it was found that multiple-type HPV infections had a significantly higher prevalence than those with single-type HPV infections in the age group up to the age of 34 , which means in younger women, but statistical analysis did not confirm a significant difference between women with single and multiple-types of HPV infections in relation to the cytological finding. It is still not clear whether in women with multiple- type HPV infections exists a competitive or cooperative interaction among the co-infecting genotypes that several studies suggested (10,12,13,16). For instance, Trottier et al. demonstrated that HPV51, 52, 56, and 58 types might cooperate with HPV16 to produce any squamous intraepithelial lesion or cancer (17). In this study, HPV co-infection and in particular the association between 16 and 18, 16 and 33, 16 and 52 in women with hrCLwas detected. The aforementioned HPV co-infections were not detected at all in women with non-hrHPV.

In addition to the possible competitive effect among HPV genotypes, the host immune response should also be considered when evaluating the increased risk for hrCL in women with multiple-type HPV infections. It has been shown that cervical HPV infection is higher in populations with impaired immune responses, such as HIV infection (18) and cigarette smoking (19). Alternatively, the weak and ineffective immunity generated by each HPV genotype may collectively provide an overall stronger immunity against HPV infection through antibody cross-reactions.

Conclusion

In this study of 212 women, HPV16 was the most prevalent type, detected in 19% of all HPV positive cases and topped the prevalence rank in ST groups with 30,5%. Other most prevalent HPV-types were: 31, 52, 59, 33 and 18 with the presence of 12,1%; 7,1%; 7,1%;

6,8%, 6,5%, respectively. Patients with multiple type HPV infections had a significantly higher prevalence than those with single type HPV in the age groups up to 34 years. HPV co-infection and in particular the association between 16 and 18, 16 and 33, 16 and 52 in women with hrCL was detected. The aforementioned HPV co-infections were not detected at all in women with non-hrHPV. The mechanisms of the possible intergenotypic competition are likely more complex and may involve multiple key stages of the HPV infection process, including binding sites/receptors, utilization of host cell organelles, synthesis of viral DNA, insertion of viral DNA into the host genome and expression of viral proteins. A better understanding of the interactions among HPV genotypes is of great interest, not only for risk assessment of hrCLs in women with multiple-type HPV infections, but also for the development of the next generation of HPV vaccines by predicting the possible emerging genotypes that compete with those targeted by current vaccines (i.e. HPV 16 and 18). Further studies including larger cohorts are needed to elucidate the underlying mechanisms.

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