Wide Spectrum Analysis of HPV Genotypes in External Anogenital Warts among Hungarian Patients

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Abstract

Background: External anogenital warts (EGW) are primarily associated with the low-risk Human papillomavirus (HPV) genotype 6 and 11, though coinfection with other low-risk and oncogenic high-risk HPV genotypes also occurs. Though the many studies about HPV-associated disease, the epidemiology, and the prevalence of HPV genotypes associated with EGW are not well characterized. The objective of our study was to determine the incidence of HPV genotypes in a Hungarian cohort diagnosed with EGW.

Methods: A total number of 94 patients’ archived formalin-fixed paraffin-embedded (FFPE) blocks were enrolled in our retrospective study. HPV genotypes were determined, applying HPV Direct Flow Chip Test.

Results: The overall prevalence of HPV DNA in EGW cases was 100%. 72.34% of the cases were mono while 27.65% were multi infections. Cumulative prevalence of HPV 6 and 11 was 98.93%; while a total of 19.4% of the cases occurred with at least one high-risk genotype. The most frequent HPV genotypes were HPV 6 (86.17%), 11 (12.76%), 42 (6.38%) followed by HPV 40, 56, 59, 66, 73 (3.19%).

Conclusion: Data of our study could provide invaluable information concerning the relative frequencies of HPV types in the Hungarian population, enabling improved assessment of the actual and future efficacy of vaccination programs and forecast changes in infection patterns.

Background

Human papillomavirus (HPV) infection is responsible for the development of external anogenital warts (EGW) often referred to as external condyloma acuminata (Fig. 1). External anogenital warts rank among the most frequent sexually transmitted diseases, approximately 65% of people who have sex with an infected partner will develop warts themselves [1, 2]. There have been over 100 types of HPV identified with around 40 strains that are known to affect the anogenital area [3]. Though primarily (90%) HPV genotypes 6 and 11 are known to be associated with external anogenital warts, combined infections with other low-risk (LR) and high-risk (HR) HPV types also occur [4–9]. People who are between 18 to 59 years of age are most commonly affected, with the highest frequency reported of 20–35 years. In adolescents, external condyloma acuminata are the most common clinical manifestations of HPV infection and up to 83% of females with external anogenital warts or a history of external anogenital warts have concomitant cervical HPV infection [4]. It is also indicated that patients with simultaneous infection are at higher risk of developing more serious diseases like cervical, penis, and anal cancer [6]. Besides, around 20% of people with EGW will present other sexually transmitted diseases (STDs) [10]. Considering these, patients with a diagnosis of EGW should be regarded as part of the high-risk population and optimal follow-up should be administered [6, 11]. These patients also take serious risk in the transmission of HPV and other STDs in the population. Some cases of EGW spontaneously resolve, though most patients require treatment [12]. Therapy options include home-based treatment (chemotoxic agents or immunomodulatory therapy) and clinician-administered therapies (cryotherapy, electrotherapy, laser and surgery); however, these treatments can solve the clinical problem, do not eliminate the basic viral cause.
Furthermore, depending on the applied method, the recurrence could be as high as 30–65%; considering these, primary prevention by HPV vaccines must be highlighted. Since external anogenital wart is not a reportable disease, its incidence is difficult to estimate. Nevertheless, based on the results of systematic reviews, 9 to 13 percent of the global population is infected; the regional distribution of new cases of anogenital warts/100,000 population/year is approximately 101 to 205 in North America, 118 to 170 in Europe, and 204 in Asia. The distribution of the different HPV genotypes varies across different populations and geographical regions. Data concerning the incidence and persistence of HPV genotypes among patients diagnosed with EGW, in different geographical regions are limited. Since such data are not yet available from Hungary, in a retrospective study, we aimed to determine the occurrence of HPV genotypes in patients diagnosed with EGW, applying a method being sensitive to 36 different genotypes (18 LR, 18 HR) of HPV. In our viewpoint, our data could provide invaluable information concerning the relative frequencies of HPV types in the target population, enabling improved assessment of the actual and future efficacy of vaccination programs and forecast changes in infection patterns.

**Methods**

**Study population**

94 patients (n = 66 females; n = 28 males) were recruited in our retrospective study. The residence of the patients mostly covers the South-West part of Hungary. The study was performed based on females and males that attended the Departments of Obstetrics and Gynaecology, Urology and Department of Dermatology, Venereology, and Oncodermatology at the University of Pécs, between 2004 to 2020, due to routine gynecological and urological screening, or because of symptoms of external anogenital warts. Patients data (e.g., age at diagnosis, area of residence, date of sample collection, history of prevalence of EGW, history of treatments, history of smoking habits, clinical pathology, morphology of the lesions, and sampling sites) were extracted from medical records. The study was approved by the clinical research ethics committees of the University of Pécs (8215-PTE 2020).

**Sample preparation**

 Archived hematoxylin-eosin stained slides of patients, earlier diagnosed with EGW, were revised. Based on the reassessment, a total number of 94 patients’ archived formalin-fixed paraffin-embedded (FFPE) blocks were enrolled in our study. The slides and FFPE blocks have been stored, and the revision was performed at the University of Pécs, Department of Pathology. The FFPE blocks were further processed for HPV genotype analysis.

**HPV genotyping**

Determination of HPV genotypes were accomplished by the HPV Direct Flow Chip Test (Master Diagnostica, Granada, Spain). The test allows the qualitative detection of 36 different genotypes of HPV (high-risk HPV: HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82; low-risk HPV: 40, 41, 42, 43, 44, 54, 55, 57, 61, 62, 67, 70, 71, 72, 74, 79).
HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 67, 69, 70, 71, 72, 81 and 84) based on direct PCR from crude-cell extracts, automatic flow-through hybridization and colorimetric detection. All the virus types are determined separately with the exception of the low-risk 44/55 and 62/81 genotypes, that are identified in clusters.

The test was performed according to the manufacturer's instructions. In brief; 3 pieces of 10 µM thick sections were treated with 400 µl of mineral oil then incubated at 95 °C for 2 minutes. After removing the mineral oil, 60 µl lysis buffer with 1.5 µl DNA release (Master Diagnostica, Granada, Spain) was added to the samples and incubated at 60 °C for 30 minutes, followed by inactivation at 98 °C for 10 minutes. To lyophilized PCR mix, 3 µl of crude cell extract and 27 µl DNase/RNase free water was added. The lyophilized PCR mix contained PCR buffer, dNTP(U/T), DNase/RNase free water, biotinylated primers, DNA polymerase, and UNG. The primers included, are specific for the amplification of a fragment of the L1 region of the HPV genome. Besides, primers for the amplification of the human beta-globin gene are included and used as an internal control for the PCR reaction. The amplification cycling condition in IANLONG PCR Thermal Cycler (Genesy 96T) were the following: pre-incubation at 25 °C for 10 min then 94 °C for 3 min. 15 cycles of denaturation at 94 °C for 30 s, annealing at 47 °C for 30 s and elongation at 72 °C for 30 s; 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and elongation at 72 °C for 30 s and final elongation at 72 °C for 5 min; followed by cooling at 8 °C. Amplicons were denatured at 95 °C for 10 min then cooled on ice for 5 min.

The full hybridization process was performed semi-automatically in hybriSpot (HS12) following the instructions provided by the wizard of the system. The management of the samples, the capture of images, and the analysis and report of the results were performed by hybriSoft software.

Statistics

The overall prevalence of HPV genotypes was calculated. The distribution of specific HPV genotypes was expressed as the proportion of HPV DNA positive specimens among all cases of EGW. Qualitative variables were studied using the 2-sided Chi test or Fisher's exact test as appropriate. Quantitative data are expressed as mean (± SD) and range. P values < 0.5 were considered to be statistically significant.

Results

Patients characteristics.

A total of 66 females (mean age 36 ± 16.9 years; median age 29.5 years; range 15–68 years) and 28 males (mean age 37.5 ± 18.46; median age 32.5 years; range 21–77 years) were enrolled; all patients provided positive β-globin results suggesting that the samples DNA amount were suitable for PCR analysis. All patients attended to be HPV positive and all the HPV genotypes were determined. The patient characteristics are presented in Table 1. Among females, 30.30% of all cases were clinically recurrent disease. We didn't find an association between the incidence of clinically recurrent cases and the ages at the diagnosis. Data according to the type of EGW diagnosis (clinically recurrent or new)
weren't available among male patients. At the time of diagnosis, 9.09% of females with EGW were pregnant. The EGW lesions mostly appeared as multiplex forms, 66.60% and 64.20% in case of female and male patients, respectively. Data on smoking habits were only available among females with the exclusion of 5 cases; according to these, 44.26% of the females were smoking at the time of the diagnosis (Table 1). Comparing the 27 smoker patients to the 27 non-smoker patients, we identified 13 clinically recurrent cases and 14 new cases in the smoker's group. When we compared the findings with the non-smoker's group, only 6 cases were found with clinical recurrence and 21 with newly diagnosed lesion; association between the smoking habit and clinically recurrence of the disease showed a significant relationship (p = 0.0239, at p < 0.5).

HPV prevalence.

HPV genotype classification was based on the HPV Direct Flow Chip Test's (Master Diagnostica, Granada, Spain) description. HPV DNA was detected in all of the 94 EGW cases and genotypes were also determined in every case. From 94 cases 68 (72.34%) appeared with 1 HPV genotype, while in 26 cases (27.65%) multi infections were found; out of these 26.90% occurred with > 2 HPV genotypes. Low-risk HPV monoinfection appeared with 72.34%. Seventy-six (80.80%) of 94 cases indicated infection with ≥ 1 low-risk genotype, without high-risk genotype, while ≥ 1 low-risk with or without high-risk genotype was found in a total of 94 samples (100%) (Table 2). According to our results, none of the cases occurred with ≥ 1 high-risk genotype without a low-risk genotype, whereas a total of 18 (19.14%) samples demonstrated coinfection with low-risk and with high-risk genotypes. Out of these 18 cases, 14 (14.89%) were infected with 1 high-risk HPV genotype, while in 4 (4.25%) cases we could detect more than one high-risk genotype (Table 2). Comparing multi infections to mono infections in the view of mean ages, we found, that multi infections were more frequent at younger ages, 34.46 compared to 38.94 (data are not significant; p = 0.276; at p < 0.05) years. The presence of ≥ 1 high-risk genotype was as common among males as among females (p = 0.22; at p < 0.05) and occurred as frequently in the new cases as in the clinically recurrent cases (p = 0.19; at p < 0.05). However, the Chi test was not significant, our results indicated, that such a pattern, as the proportion of ≥ 1 high-risk genotype was more frequent at ages under 30 (Table 3).

Occurrence of the specific genotypes.

Out of the 36 tested (18 low-risk, 18 high-risk) HPV genotypes, 10 low-risk, and 11 different high-risk HPV genotypes were detected in EGW samples (Table 4). The most frequently identified HPV genotypes were as follows, by decreasing frequency: HPV 6 (86.17%), HPV 11 (12.76%), HPV 42 (6.38%) and HPV 40, HPV 56, HPV 59, HPV 66, HPV 73 (3.19%). DNA of the low-risk HPV 6 and HPV 11, alone or in combination with each other, was found in 72.34% of the cases; the overall prevalence of HPV 6 and 11 genotypes alone or in combination with any other HPV types (low-risk and/or high-risk) occurred to be 98.93%. DNA
of the low-risk HPV 40 was the only type that was observed independently from HPV 6 and/or HPV 11 in one case; whereas DNA of the other low-risk HPV types including 42, 44/55, 54, 67, and 62/81 were always found in combination with HPV 6 and/or HPV 11, representing 7.42% of frequency in all EGW cases (Fig. 2). The most common high-risk HPV genotypes as HPV 56, 59, 66, and 73 were detected at the same frequency, 3.19% of all EGW cases, yielding to the overall prevalence of 54.54% out of all high-risk infections (number of total high-risk infections = 22) (Table 4). DNA of HPV 16 and HPV 18, occurred at the same frequency as 2.12% of all EGW cases, yielding to the overall appearance of 18.18% among the high-risk positive infection types. Out of the detected 11 high-risk types among EGW cases, 10 (90.90%) types (HPV 16, 18, 39, 51, 52, 56, 66, 68, and 73) were found among females, while 4 (36.36%) types (HPV 18, 53, 56 and 73) were observed among males, yielding to a 70% discrepancy between female and male patients. In contrast, excluding the three overlapping genotypes (HPV 18, 56, and 73), the discrepancy between male and female cases was only 10%; the DNA of HPV 53 was the only one that was just observed among male EGW cases. However, the results are not significant, this pattern indicates, that more kind of high-risk HPV DNA were represented among female than male EGW cases (p = 0.46 at p < 0.05). In the case of the low-risk genotypes, 60% of the detected HPV types shared homology (HPV 6, 11, 40, 42, 54, 67) among female and male EGW cases, while 40% of the genotypes (HPV 44/55 and 62/81) were only found among females.

Discussion

External anogenital warts are the most common, macroscopically visible, clinical manifestation of HPV infection of the lower genital tract. The vast majority of the cases were monoinfection of the low-risk HPV genotypes 6 and 11. It has been proposed that low-risk HPV genotypes do not integrate their DNA into the chromosomes of the infected cells, hence such low-grade lesions have a low-risk of progression to malignancy. However, around 19–33% of the EGW cases are coinfected with oncogenic, high-risk HPV genotypes [6, 22]. While most of the studies and screening programs focus on the HPV related malignant disease, the epidemiology of external anogenital warts are not well characterized; data about the prevalence of different HPV genotypes related to EGW, especially in Mid-European countries, are underrepresented and incomplete [22]. The present study aimed to determine the prevalence of different HPV genotypes in surgical external anogenital wart samples’ of a cohort, diagnosed with hard-to-treat EGW. Formalin-fixed paraffin-embedded samples of 94 patients (66 females and 28 males) with EGW were recruited in the study. The explanation of the significantly smaller sample size of the men’s group could be due to the different attitudes of visiting doctors. Unlike females who routinely take part in screening programs and undergo follow-up with gynecologists, males usually seek consultation with specialists if they present symptoms. Asymptomatic individuals often not identified, representing an invisible proportion for healthcare systems. The observed overall 100% prevalence of low-risk HPV infection indicated that EGW lesions never appear in the absence of low-risk HPV infection. According to our results, among the examined Hungarian patients the most common HPV genotypes were HPV 6 and 11. The vast majority of the samples, 72.34%, were diagnosed with monoinfection of HPV 6 and 11.
genotypes, while these two genotypes either alone or in combination with other HPV genotypes, occurred in 98.93% of the cases. Apart from the applied method, data regarding the prevalence of HPV 6 and 11 genotypes are comparable to the data reported from other European countries. By applying a highly sensitive method we were able to identify a wide spectrum of HPV coinfections. In our study, 27.65% of the observed cases were multi infections and out of these cases, 26.90% were co-infected with more than two HPV genotypes. There were no significant differences in the presence of multi infections among females and males. Comparing multi infections and mono infections by the mean ages, we could conclude, the proportion of multi infections were more frequent at lower mean age, as 34.46 compared to 38.94 years.

Focusing on high-risk genotypes we could conclude that oncogenic, high-risk HPV genotypes never occurred as mono infection. Our study revealed, that out of the 27.65% coinfected samples 19.4% of the cases occurred with ≥ 1 high-risk HPV genotype. Although data were not significant, by analysing the prevalence of high-risk genotypes according to different age distributions, in line with other studies, we found that the proportion of ≥ 1 high-risk genotype is more frequent at ages under thirty [4]. This could be explained by lifestyle attributes. Furthermore, our results also indicated more HPV genotypes among female patients. Interestingly, we observed slight differences according to the prevalence of most frequently appearing high-risk genotypes compared to the data reported by other European countries; instead of HPV 16 being the most common high-risk type, HPV 56, 59, 66, and 73 occurred to be the most frequent genotypes presenting with the same prevalence of 3.19% each, of all EGW cases [21, 22]. In the case of the other tested genotypes, the prevalences are comparable to the data reported in European countries; the observed slight differences could be mostly attributed to the variation in the methods used for HPV detection.

Nowadays, wide spectrum of diagnostic HPV tests are available on the market, used in routine diagnostics, such as INNO-LiPA, Linear Array HPV Genotyping test, PapilloCheck HPV test, Digene Hybrid Capture 2, CLART HPV2, etc. Nevertheless, these methods show wide differences regarding genotype-specific sensitivity and sample types that are validated in the applied method [29–32].

In our study, a sensitive, genotype-specific method, HPV Direct Flow Chip Test was used. The technology is based on the amplification of a short DNA fragment of HPV L1 ORF, enabling the use of FFPE samples. The test is CE IVD marked in compliance with European Union diagnostic medical device manufacturing standards. Though the most acceptable sampling method from the external lesions is using cytobrush, it is reported that these lesions often don’t provide proper cell retrieval, resulting in insufficient material for genotyping [21]. Considering these, it is an important advantage, that the applied method is validated for FFPE sample material as well, providing suitable material for the analysis. The sensitivity and specificity of the applied method had been proved in independent studies [29–31]. Regarding these studies, although the HPV Direct Flow Chip showed a very high genotype-specific concordance with the other methods (Linear Array, Hybrid Capture 2, INNO-LiPa systems) in the pair-matched results, the HPV Direct Flow Chip yielded significantly higher rates of HPV infection than other methods used in the study,
especially in case of FFPE samples, moreover, the HPV Direct Flow Chip test proved more effective than other HPV detection systems in samples with low viral load [29].

Based on the analysis of Mayenaux and Chaturvedi the incidence of HPV infection could be kept in check; though the continuously increasing number of risk factors, as the growing number of lifetime sexual partners, earlier sexual debut (< 16 ages), homosexual relationships, history of other STDs, smoking and human immunodeficiency virus (HIV), could only be counteracted by screening programs and vaccination [15].

Currently, 3 licensed HPV vaccines are available using L1 capsid antigens of 2, 4, or 9 HPV genotypes. All the 3 vaccines include HPV 16 and 18, which cause the majority of HPV related cancers. While the quadrivalent (Gardasil 4) and nonavalent (Gardasil 9) also target low-risk types HPV 6 and 11, which are primarily associated with external anogenital warts. In addition, the nonavalent vaccine includes 5 other high-risk HPV types above, such as HPV 31, 33, 45, 52, and 58. A very high efficacy of the quadrivalent and nonavalent HPV vaccines against HPV 6 and 11 associated disease was reported in multiple randomized, controlled trials [34–36]. There is accumulating evidence that population-based vaccination targeting HPV 6 and 11 as well can result in dramatic declines in genital warts incidence [33, 35]. Regarding our results, we found that the prevalence of HPV 6 and/or HPV 11 was 98.93% among all EGW cases indicating a potentially great benefit from the vaccine. Though it must be highlighted that out of the 11 detected high-risk genotypes the nonavalent vaccine covered only 3 genotypes, while out of the 10 detected low-risk genotypes, it covered 2 genotypes, suggesting the importance of follow up of patients diagnosed with EGW. In Hungary, the bivalent vaccine has been available since 2007, while the quadrivalent vaccine, that was available from 2006 was replaced with the nonavalent vaccine in 2015. As part of the national immunization program, in 2014 the bivalent HPV vaccine was introduced to 13-year-old girls by a school-based vaccination program. In 2018 the bivalent vaccine was replaced and since then the nonavalent vaccine has been applied, benefiting protection against EGW as well. The acceptance of the vaccine is increasing among girls. It was around 80% in 2019, based on the result of the State Public Health and Medical Officer. It is a great advance, that males at 13 years of age have also been recruited in the national HPV vaccination program from 2020. Though the attitude seems positive, educating the population could further improve the coverage of the vaccine [37].

**Conclusion**

The published data on the type-specific HPV associated disease in the Central and East European countries are scarce. Our retrospective study is the first that provides data on the prevalence of different HPV genotypes among Hungarian patients diagnosed with EGW. While most of the studies have analysed only small numbers of genotypes, our wide spectrum analysis of HPV genotypes provides reliable data regarding the prevalence and distribution of HPV genotypes in EGW cases, hence enabling improved assessment of the actual and future efficacy of vaccination programs and forecast changes in infection patterns.
Limitation

Although our results show high accordance with those of other studies on HPV and EGW, the relatively small sample size and limited clinical data of the male patients made the statistical comparison difficult.

Abbreviations

EGW: external anogenital warts; FFPE: formalin-fixed paraffin-embedded; HR: high-risk; HIV: Human Immunodeficiency virus; HPV: Human papillomavirus; LR: low-risk; STD: Sexually transmitted disease

Declarations

Ethical approval

This study received ethical approval from the Clinical Research Ethics Committees of the University of Pécs (8215-PTE 2020).

Consent for publication

Not applicable

Acknowledgements

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

OR, AO and KK initiated and designed the study. OR and EM analysed the data. AO, KK EK and TT were responsible for the reanalysis of the archived hematoxylin-eosin stained slides. OR was responsible for writing up and all (OR, AO, EM, EK, TT, KK) were involved in the discussion of the results. All authors have read and approved the final manuscript.
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**Tables**

Due to technical limitations, table 1 to 4 is only available as a download in the Supplemental Files section.