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Detection of high risk human papillomavirus types 16 and 18 in immune competent Egyptian patients with plantar and genital warts

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ABSTRACT

Objectives: Human papilloma viruses have been identified in cutaneous and genital warts with minimal reports targeting, in particular, the association of high risk HPV-16 and 18 in Egyptian patients with genital warts, together with the absence of studies investigating these 2 viruses in plantar warts. Aim: To assess the impact of the virus type and investigate the association of high risk HPV-16 and 18 in plantar and genital warts of immune competent Egyptian patients. Patients and Methods: Fifty four Egyptian patients (33 with plantar warts and 21 with genital warts) were randomly included in this work. Highly sensitive polymerase chain reaction (PCR) with two different set of primers was used for the detection of each of the highly oncogenic potential viruses HPV-16 and HPV-18. Results: Both high risk HPV-16 and 18 were detected in two patients with plantar warts using the two different set of primers. The incidence of presence of HPV-16 and18 in genital warts was six times higher than that of plantar warts with a statistically significant difference between both groups (P-value < 0.05). Conclusion: This is the first study to detect high risk HPV-16 and 18 in plantar warts in Egyptian patients. The percentage of these viruses in genital warts was higher than reports by previous studies in literature.

Key words: Genital warts, human papilloma virus 16, human papilloma virus 18, immune competent, plantar warts.

Introduction

Warts are benign proliferations of stratified squamous epithelium caused by the human papilloma virus (HPVs). A majority of the HPVs infected the normal skin as well as immune compromised individuals. It appeared that most HPVs establish a latent infection of the skin as normal flora residing in hair follicles (Jenson A. 2001; Payal R. 2006). More than 200 types of HPV had been recognized on the basis of DNA sequence. Specific types tend to show some tissue tropism, depending on the type of epithelium infected; HPV types are often referred to as cutaneous or mucosal types (Gómez D. 2007). Within each of these HPV groups, individual viruses were designated high risk or low risk according to the propensity for malignant progression of the lesions that they caused. Most HPVs were low risk and produced localized benign warts that did not undergo malignant progression even if left untreated (Bonnez W. 2000). The majority of HPVs were associated with cutaneous warts (plantar warts, common warts and flat warts). Plantar warts were commonly caused by HPV type 1 and less commonly by 2 and 4. About 40 HPVs infected the genital tract through sexual contact which were further divided into high and low-risk types. Low-risk HPVs include types 6, 11, 42, 44, and 54 that were associated with condylomata accuminata and low-grade dysplasia. High-risk HPV types include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 70, and cause anogenital cancer. A role for HPV in non-melanoma skin cancer (NMSC) has been proposed (De Villiers E. 2004 and Reuschenbach M. 2011). In particular, HPV 16 and HPV 18, were known to cause up to 95% of cervical cancers, and new studies showed that they might be linked to oral cancer, too (Sumino Y. 2004 and Fabbrocini G. 2009). To the best of our knowledge, no much data had been reported before on high risk HPV16/18 in immune competent Egyptian patients with genital warts. Moreover, to date, data on their association with plantar warts are minimal. Therefore, the aim of this study was to investigate the presence or absence of high risk HPVs types 16 and 18 in plantar warts as well as in genital warts of immune competent Egyptian patients.

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Patients and Methods:

Patients:

This pilot study included 54 immune competent Egyptian patients (38 males and 16 females) with either plantar or genital warts that were randomly collected. They were divided into 2 groups: group (I) included 33 patients with plantar warts and group (II) included 21 patients with genital warts. The duration of the disease was 2 weeks to 10 years in group I and 1 month to 10 years in group II with a mean ± standard deviation (SD) of 4.2 ±3.8, 5.1±3.2 years, respectively. Patients with signs of any inflammation, infection, or an associated disease, pregnant or lactating females, and immune suppressed patients, as well as patients with genital warts who had recent sexual contact were excluded. All patients were recruited from the Dermatology outpatient clinics of the National Research Center and Al-Azhar University Hospitals. An informed consent was taken from all patients before participating in this work. The study was approved by the ethical committee of the National Research Center, Giza, Egypt.

Methods:

All patients were subjected to detailed history taking, general and dermatological clinical examination for the type of warts, site, size, number of lesions, and associated tenderness or pain.

Skin sampling:

Two skin biopsies were obtained from each patient. Biopsies were taken from each area away from the genital mucosa in case of genital warts from the plantar surface of the foot in case of planter warts. The biopsies were done under local anesthesia using a 3 mm punch biopsy of the selected warts. The first biopsy was fixed in 10% natural buffered formalin and embedded in paraffin. Four micron thick sections from the paraffin embedded biopsies were stained by Haematoxylin and Eosin to verify the clinical diagnosis of warts, and to detect any malignant changes in the lesions. The second biopsy was stored in normal saline for DNA extraction PCR.

DNA Extraction:

DNA was extracted from the tissues using the method of (Sambrook J. 1989). This method provides a pure preparation of under graded DNA in high yield.

Polymerase chain reaction (PCR) for human papilloma virus 16/18:

Two different set of primers were used in two different PCR tests for the detection of each of HPV16 and HPV 18. The sensitivity assays were performed for both tests by amplification of HPV- positive cells (SiHa cells of HPV-16 and HeLa cells for HPV-18). The first PCR test using primers comprising the type-specific open reading frame (ORF) E6 regions of HPV16 & 18 (Table 1) (Burmer G. 1990). PCR reaction containing 4.5 mM magnesium chloride, 200 µM of dNTPs, 5 units of Taq DNA polymerase (Gibco BRL, Gaithersburg, Md.), and 6 µM of each primer in a total volume of 25 µl. Thermal cycling protocol comprised: initial denaturation at 95°C for 5 minutes, followed by 30 cycles each includes denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes and a final extension step at 72°C for 10 minutes. Amplification product was resolved on 2% agarose gel electrophoresis. A fragment of 109 base pair (bp) indicated successful amplification of HPV 16 (Figure 1), and a fragment of 334 base pair (bp) of HPV 18 (Figure 2).

The second PCR test used type-specific primers for each of HPV16 (Fontaine V. 2007) and HPV 18 (Baay M. 1996) (Table 1). PCR reaction contained 4 mM magnesium chloride for (HPV 16) and 2.5 mM for (HPV 18), 200 µM of dNTPs, 5 units of Taq DNA polymerase (Gibco BRL, Gaithersburg, Md.), and 50 pmol of each specific primer, 10ul of DNA in a total volume of 50 µl. Thermal cycling protocol for HPV16 comprised: initial denaturation at 95°C for 5 minutes, followed by 3 5 cycles each includes denaturation at 95°C for 45 sec, annealing at 54°C for 45 sec and extension at 72°C for 1 minute and a final extension step at 72°C for 10 minutes. The Thermal cycling protocol for HPV18 the same as HPV16 except the annealing step was performed at 51°C for 30 sec. Amplification product was resolved on 2% agarose gel electrophoresis. A fragment of 105 base pair (bp) indicated successful amplification of HPV 16 and a fragment of 115 base pair (bp) of HPV 18 (Figure 3).
Figure (1) Agrose gel electrophoresis of HPV-16 amplicons (Test I).

Fig 1: Agarose gel electrophoresis of HPV-16 amplicons (Test I). Lanes 2, 4 and 7 represent amplified product of 109 base pair (bp). Lane 1 represents 100bp molecular weight marker.

Figure (2) Agrose gel electrophoresis of HPV-18 amplicons (Test I).

Fig 2: Agarose gel electrophoresis of HPV-18 amplicons (Test I). Lanes 1, 2 and 3 represent amplified product of 334 base pair (bp). Lane M represents 100bp molecular weight marker.

Figure (3) Agrose gel electrophoresis of HPV-16 and HPV-18 amplicons (Test II).

Fig 3: Agarose gel electrophoresis of HPV-16 and HPV-18 amplicons (Test II). Lanes 3, 4 and 5 represent amplified product of 115 base pair (bp) indicating successful amplification of HPV 18. Lanes 7, 8 and 9 represent amplified product of 105 base pair (bp) indicating successful amplification of HPV 16. Lane M represents 50bp molecular weight marker.
Table 1: Oligonucleotide primers used to detect human papilloma virus-16 and 18 DNA by polymerase chain reaction in two different tests.

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Primer sequence of (PCR Test I)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 – upstream</td>
<td>5`ATT AGT GAG TAT AGA CAT TA-3</td>
<td>109</td>
</tr>
<tr>
<td>16 – downstream</td>
<td>5`GGC TTT TGA CAG TTA ATA CA-3</td>
<td>109</td>
</tr>
<tr>
<td>18− upstream</td>
<td>5`ACT ATG GGG CGC TTT GAG GAT CCA-3</td>
<td>334</td>
</tr>
<tr>
<td>18− downstream</td>
<td>5`GGT TTC TGG CAC CGC AGG CA-3</td>
<td>334</td>
</tr>
<tr>
<td>HPV type</td>
<td>Primer sequence of (PCR Test II)</td>
<td>Product length (bp)</td>
</tr>
<tr>
<td>HPV-16 (1)</td>
<td>5<code>TTT GGT CTA CAA CCT CCC CCA GGA-3</code></td>
<td>105</td>
</tr>
<tr>
<td>HPV-16 (2)</td>
<td>5<code>TTC TTT AGG TGG AGG GAT ATG-3</code></td>
<td>105</td>
</tr>
<tr>
<td>HPV-18 (1)</td>
<td>5<code>CCC TGG AGG TAA ATT TTT GG-3</code></td>
<td>115</td>
</tr>
<tr>
<td>HPV-18 (2)</td>
<td>5<code>CAC GCA CAC GCT TGG CAG GT-3</code></td>
<td>115</td>
</tr>
</tbody>
</table>

Statistical analysis:

Data were analyzed using SPSS ver. 15.0.1 for windows (SPSS, Chicago, IL, USA). Mean and Standard deviation were used to describe continuous data. Comparison between two means was done using an independent samples Student’s t-test. Categorical data was presented as number and percentage. Chi square was used for comparison between two categorical variables. Odds ratio was used to study the degree of association and estimation of risk. P< 0.05 was considered statistically significant.

Results:

Fifty four Egyptian patients were enrolled in this study, group I included 33 patients (61.1%) with plantar warts. Their age ranged from 8 to 38 years with a mean ±SD of 22.1±5.6 years. Twenty patients of them were males (60.6%) and 13(39.4%) were females. Male to female ratio was 1.5:1. The plantar wart lesions were single in 10 patients (30.3%) and multiple in 23 patients (69.6%). Plantar wart lesions were either painful or tender in all patients.

Meanwhile, group II included 21 patients (38.8%) with genital warts. Their age ranged from 11 to 60 years with a mean ±SD of 33.8 ±12.2 years. Eighteen patients were males (85.7%) and 3(14.3%) were females. Male to female ratio was 6:1. The genital wart lesions were multiple in all patients. Lesions were associated with bleeding on touch in 5 patients (23.8%) and with itching in 7(33.3%) patients.

Histopathological Examination:

Routine histopathological examination with Hematoxyline and Eosin stain showed typical changes of plantar and genital warts. None of the lesions showed any changes suggestive of malignant changes, dysplasia or nuclear atypia.

Polymerase chain reaction for human papilloma virus-16 and-18 in group I patients (plantar warts):

In group I (plantar warts): seven patients (21.2%) were positive for HPV-16 patients in the first PCR test while 4(12.1%) patients were positive in the second PCR test. Only 2 patients (6.1%) were positive in both tests. While for HPV-18, the first PCR test showed that 9(27.2%) patients were positive, however in the second PCR test: 3(9.1%) patients were positive. Only 2(6.1%) patients were positive for HPV-18 in both tests. The 2 patients that were positive for both HPV-16 and 18 were the same patients. Both were females aged 20 years. The duration of warts in the first patient was 2 years while it was 6 months in the second. Both patients had multiple plantar warts.

Polymerase chain reaction for human papilloma virus-16 and 18 in group II patients (genital warts):

Group II (genital warts) patients were positive for HPV-16 in the first PCR test in 5(23.8%) patients while the second PCR test was positive in 4(19%) patients. Only 2(9.5%) patients were positive for the virus in both tests. Both of these 2 patients were males. The first was 37 years old with multiple genital warts associated with bleeding on touch and the duration of lesions was 6 years while the second patient was 23 years old, with multiple genital warts and the duration of lesions was 2 years.

As regards for HPV-18, the first PCR test showed that 7(33.3%) patients were positive for the virus while the second PCR test was positive in 5(23.8%) patients. Only 4 (19%) patients were positive for HPV-18 in both tests. The 4 positive patients were 2 females and 2 males. The 2 male patients were aged 33 years with multiple genital warts associated with itching in one of them. The duration of lesions was 1 year in the first patient and 7 months in the second. The 2 female patients aged 25 years and had multiple genital warts associated with itching. The duration of lesions was 1 year and 9 months respectively.
The relation between plantar and genital warts with human papilloma virus-16 and 18:

The incidence of presence of oncogenic HPV-16 or 18 in genital warts was higher than that of plantar warts. Group II patients (genital warts) had 6(28.6%) patients positive for HPV-16 or 18, while group I patients (plantar warts) had only 2(6.1%) patients positive for HPVs-16 and 18 with a statistically significant difference between both (P-value< 0.05) (Table 2). The risk of presence of oncogenic HPV-16 and 18 (Odds ratio) was 6 times in cases of genital warts compared to that of plantar warts: Odds ratio= 6(95% confidence interval) (Table 3).

Table 2: Relationship between type of wart and human papilloma virus positivity (regardless of human papilloma virus type) using Chi-square test.

<table>
<thead>
<tr>
<th>Wart Type</th>
<th>Positive for HPVs 16 and 18</th>
<th>Negative for HPVs 16 and 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percentage</td>
</tr>
<tr>
<td>Genital</td>
<td>6</td>
<td>28.6</td>
</tr>
<tr>
<td>Plantar</td>
<td>2</td>
<td>6.1</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>14.8</td>
</tr>
</tbody>
</table>

P= 0.04 (significant)

Table 3: Odds Ratio for genital / plantar cases.

<table>
<thead>
<tr>
<th>Odds ratio for positive cases</th>
<th>value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>(genital / plantar)</td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>positive cases</td>
<td>4.5</td>
<td>1.0</td>
</tr>
<tr>
<td>negative cases</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>No of valid cases</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

Relationship between number of warts per patient, associated symptoms, age of patients and human papilloma virus positivity (regardless of human papilloma virus type):

The 8(18.1%) positive patients for HPVs (either 16 or 18) had multiple lesions but there was no significant difference between positive and negative cases as regards multiplicity of the lesions (P-value>0.05). On comparing the 8 positive patients for HPVs to the negative cases, there was also a non-significant difference between positive and negative cases as regards any associated symptoms (P-value>0.05). Additionally, no significant difference was detected between the 8 positive patients for HPVs and the negative cases as regards the age (mean ±SD was 27±6.8 years; 36.6±11.0 years respectively, P value=0.5).

Discussion:

The diagnosis of HPV infection is an evolving field. DNA testing had greatly expanded the options available for the detection and study of HPV disease. Most of the studies conducted on cutaneous warts had been carried out more frequently in immune suppressed patients (Fabbrocini G. 2009). In this study, we hypothesized that due to racial differences and different assays used, there could be changes in the incidence of HPV-16 and 18 in Egyptian patients with genital warts compared to those reported in other studies. This together with the rarity of studies reporting the association of these two viruses in plantar warts led us to investigate them in immune competent Egyptian patients using the PCR technique.

The histopathological examination in the current study indicated that the lesions were either typical plantar warts or condyloma accuminata. None of the specimens examined revealed any dysplastic changes. Our findings were corresponding to those of (Brown A. 1999) where none of the apparently healthy patients examined in their study revealed any signs of dysplasia in their pathology.

Polymerase chain reaction was used in the current study to detect HPV types 16 and 18 in all lesions. For the accuracy of the results, it was repeated twice for all samples using two different set of primers to confirm positive as well as negative results. Only samples that were positive using both set of primers were considered positive. Most of the studies done in this field repeated the PCR test using a second different primer only to those samples that gave positive results in the first PCR test reference (Potocnik M. 2007).

In the present study, out of the 21 patients with genital warts, high risk HPV-16 was detected in 2(9.5%) male patients while type 18 was detected in 4 patients (19.1%), (2 males and 2 females) and both types were detected in 6 patients (28.6%).

Reviewing published data in literature, revealed that Paul K. 2009 reported that HPV-16 and 18 were detected in 8 patients (6.1%) and 4 patients (3%) respectively out of the 130 Chinese male patients with anogenital warts who were examined using the PCR test. Potocnik M. 2007 detected HPV-16 in 2 male patients (3.6%) with genital warts out of 55 examined cases from Slovenia. None of the lesions in their study demonstrated HPV-18. Brown A. 1999 also demonstrated a high percentage of positivity as regards HPV-16 and 18 in both immune competent and immune compromised patients. They used a scoring system for positivity of
HPVs which made their results quantitative rather than qualitative as compared to our results. Sugase M. 1991 detected HPV-16 in 5.9% of their cases with no lesions demonstrating HPV-18 using Southern blot hybridization analysis.

The discrepancy between our findings and those of previous studies could be explained by the fact that in the present study, the detection of viruses was done using two different set of primers of two PCR tests in order to have more reliable methods for viral detection. We also used fresh frozen tissues which result in more reliable results compared with paraffin embedded tissues used in the previous studies that usually yield suboptimal results. Additionally, many of the primer pairs used in several studies either degenerate or specific ones have been used by several authors with variable results regarding the percentage of HPV detection in warts of immune competent and immune suppressed patients (Payal R. 2006).

Of special note, the present data in our study showed that the incidence of HPV-16 and 18 in Egyptian patients with genital warts appeared to be higher than that reported by previous studies (Kashima M. 2003; Lozzi G. and Peris K. 2007). This difference was actually expected, as we believe that there are racial differences and sexual behavior in any population as the number of life time sexual partners, frequency of sexual contact and age may play a role. Moreover, the large number of patients included in their studies as well as recruiting only male patients in some of them could also be considered (Potocnik M. 2007; Lozzi G. and Peris K. 2007). Existing data in literature however, do not provide a clear explanation for the observed variation in the incidence trends for HPV between different races. It is unclear whether this discrepancy is due to race-related differences in the risk of exposure to particular variants or not. Further studies on a larger scale are however, needed to confirm our findings.

To the best of our knowledge, this is the first study to detect oncogenic HPV types 16 and 18 in Egyptian patients with plantar warts as 2(6.1%) female patients out of 33 patients presenting with plantar warts had high risk HPV type 16. The same 2 female patients also had high risk HPV type 18(6.1%).

Most studies in literature detected common causative HPVs types 1, 2 and 4 in plantar warts while other studies detected rare causative HPVs like type 37 or type 60 (Kashima M. 2003). Very few studies reported the transformation of long standing plantar warts to plantar verrucous carcinoma (epitheliumacuniculatum) which is considered a slowly growing, locally invasive, well-differentiated squamous cell carcinoma (Lozzi G. and Peris K. 2007).

With improved PCR techniques the association of HPV in non-melanoma skin cancer had been documented (Reuschenbach M. 2011). No particular HPV type has yet emerged as predominant. Moreover, it is also well established that verrucous carcinomas of the vulva and the penis might occasionally develop in persisting genital warts (ZurH. 2008). Although high-risk HPV types 16 and 18 had been demonstrated in plantar and genital warts in the present study, it is unknown what percentage may progress to develop neoplastic lesions. HPV-16 and 18 might remain latent for a prolonged period of time. Patients harboring high-risk HPV might progress to invasive squamous cell carcinoma after a long latency period of 20-50 years. It had also been suggested that HPV alone might not lead to development of skin cancer but this may require other additional factors such as smoking to lead to neoplastic diseases (Jenson A. 2001).

Vaccines against HPV had been recently introduced to the national vaccination programs of several countries (Mammas I. 2008). The quadrivalent HPV vaccine had been approved for use in women and girls to prevent four types of HPV including two strains responsible of 75% of all cervical cancer. Recently it was approved for use in men and boys meaning that male patients might ask dermatologists about preventive therapy. This vaccine had also therapeutic indications not only preventive. These findings suggested that if the recently approved quadrivalent HPV vaccine proved to be effective in preventing HPV infection in males as it has proved in females, the vaccine could prevent the great majority of incidental genital warts in males (Albarran C. 2007 and Venugopal S. 2010).

To conclude, this is the first study to detect high risk oncogenic HPV-16 and 18 in Egyptian patients with plantar warts. Patients with genital warts appeared to have a higher incidence of the two viruses compared to those detected in other studies. High risk HPV-16 and 18 associated with benign proliferations of the skin, further expands the spectrum of HPV-linked lesions. It is still of critical interest to determine which HPV types are specifically associated with the development of malignant cutaneous lesions analogous to those seen in anogenital cancers. Further studies are however, required on a large scale to prove that the presence of HPV 16 and 18 that was relevant to the warts by either positive P16 staining or expression of HPV16 and 18 mRNA besides the evidence of integration of HPV 16 and 18DNA.

References


