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Human papillomavirus subtype 16 is common in Pakistani women with cervical carcinoma

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Summary

Introduction: Human papillomavirus (HPV) is recognized as a major causative agent for cervical carcinomas. Based on their oncogenic potential, HPV subtypes have been divided into high- and low-risk. In Pakistan, screening for HPV in female patients is not commonly practiced, and as a consequence, the degree of HPV prevalence and its correlation with cervical cancer is unknown.

Objective: In this study, we have attempted to estimate the prevalence of HPV infection, and also the HPV subtype profile, among Pakistani women with cervical cancer from varied geographical, racial, and social backgrounds within Pakistan.

Methodology: Women visiting two tertiary care hospitals in Karachi, diagnosed with carcinoma of the cervix within the past 15 years, were analyzed for HPV subtypes in their cancer specimens. Retrospectively, 60 paraffin-embedded cervical cancer biopsies were examined for the presence of HPV DNA. After DNA extraction from these samples, polymerase chain reaction (PCR) was used to amplify the HPV L1 gene using the consensus (general) primers, and primers specific for subtypes 16 and 18.

Results: Of the 60 samples analyzed, only one sample was HPV negative; the rest of the samples were positive for the presence of HPV. Of the 59 HPV positive samples, 56 showed the presence of HPV16 and one sample was positive for HPV18; HPV subtype could not be determined in two samples.

Conclusion: Our results show a strong relationship between HPV infection and cervical cancer among Pakistani women. These results underscore the need to implement regular HPV screening for Pakistani women. An early diagnosis of HPV infection will allow better health management to reduce the risk of developing cervical cancer.

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Introduction

The human papillomavirus (HPV) is one of the most common sexually transmitted viruses among men and women in Western countries.¹ The link between HPV infection and the development of pre-cancerous cervical, anal, and genital lesions, as well as cervical, anal, and genital cancers, is well established.² There are over 100 different subtypes of HPV, which are distinguished by variations in their genetic sequence.^{2,3} On the basis of their oncogenic potential, HPV subtypes are classified into high- and low-risk.^{4–6} Infection with high-risk HPV genotypes leads to an increased risk of cervical carcinoma. Among the HPV subtypes that infect the anogenital tract, subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, and 69 have been defined as high-risk for cervical carcinoma.^{2,3} These HPV subtypes are commonly found in anogenital cancers but typically do not cause noticeable warts; as a result, HPV infection may go undetected.^{4–6}

Cervical cancer is the second most common malignancy in women worldwide, and it remains a leading cause of cancer-related death for women in developing countries. Under the best medical facilities, at least 37.5% of the diagnosed patients die from their disease each year. This represents 2% of all cancer deaths and 18% of deaths from gynecological cancers.⁷ Cervical carcinoma is more common in Hispanic, African American, and Native American women than in Caucasian women.³ In the USA, it is the fourth most common malignant neoplasm in women, after carcinoma of the breast, colorectum, and endometrium. The incidence of invasive cervical cancer has declined steadily in the USA over the past few decades; however, it continues to rise in many developing countries. The change in the epidemiological trend has been attributed to mass screening with Papanicolaou (Pap) smears,⁸ with the help of which 500 000 new cases are diagnosed each year internationally.

In Pakistan, HPV screening is not commonly practiced. Additionally, risk factors and modes of transmission for HPV among Pakistani women have not been adequately evaluated. A major barrier in establishing the epidemiology of HPV in this country is the social taboo on all matters pertaining to sex, including sexually transmitted infections (STIs). These socio-cultural prohibitions create a substantial barrier to the investigation of issues concerning STIs. As a result, there are no or very little data available to quantify the burden of HPV and HPV-associated cervical carcinoma in Pakistan.⁹

The present study was carried out as a first step towards the compilation of HPV epidemiology in Pakistan. The intent of this work was to seek a potential relationship between HPV infection and the occurrence of cervical carcinoma. Furthermore, subtyping for certain high-risk HPV subtypes was performed to determine which were common among Pakistani women.

Materials and methods

Sample collection

A total of 60 formalin-fixed paraffin-embedded samples of cervical biopsies were collected from tertiary care hospitals in Karachi, Pakistan, namely, the Aga Khan and Civil Hospitals. The biopsies were obtained during the period of January 1991 to June 2005. The samples were from female patients

aged 20 to 60 years, already diagnosed with squamous or adenocarcinoma of the cervix. These patients visited the Aga Khan and Civil Hospitals from various parts of the country, and represented wide social and economical strata, diverse ethnicities, and varied genetic backgrounds.

Slides of all biopsy samples were reviewed by an expert histopathologist to confirm the diagnosis. Informed consent was obtained from patients for the use of their samples for research purposes.

DNA extraction

For DNA extraction from paraffin-embedded tissue, the methods of Baay et al. and Mikaelssdottir et al.^{10,11} were adopted for our experiments. To increase DNA yield and quality, the extraction procedure was modified as follows: 3–5- μm sections of paraffin-embedded cervical carcinoma tissue were cut on a microtome and tissues were deparaffinized with xylenes. Washes of 100% ethanol were used to remove traces of xylenes from the tissue. After the traces of ethanol were removed by air-drying, the tissue was homogenized and the following were added: 20 μL 20% SDS, 80 μL protein kinase buffer (0.375 M NaCl, 0.12 M EDTA, pH 8.0), and 40 μL of proteinase K (10 mg/mL); sterile water was added to a final volume of 380 μL . The mixture was incubated at 55 °C overnight to allow digestion by proteinase K.

The following morning, 100 μL of 6 M NaCl was added to the sample. The protein precipitate was removed by centrifugation for 5 min at 13 000 rpm, and the supernatant was transferred to a fresh tube. To precipitate the DNA, 1 mL of 100% ethanol was then added to the sample. The sample was centrifuged at 13 000 rpm for 5 min, and the DNA pellet obtained was washed with 70% ethanol and air-dried. The pellet was then resuspended in 50 μL of 1 \times TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) buffer and stored at –20 °C until further use.

Polymerase chain reaction (PCR)

The samples were analyzed by PCR using four sets of primers, i.e., GP5/GP6, general primers for HPV; TS16-A/TS16-B and TS18-A/TS-18B, subtype-specific primers for HPV subtypes 16 and 18, respectively;¹⁰ and PC03/PC04 for β -globin.¹¹ Sequences for the primers are given in Table 1, along with the length of respective PCR amplicons and the targeted gene. The PCR conditions for these primers were as follows.

For primers GP5/GP6, the total 25 μL PCR reaction mixture contained 5 μL sample, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM MgCl₂, 200 μM deoxynucleotide triphosphates (dNTPs), 0.4 pmol of each primer, and 0.2 U of Taq polymerase. The PCR thermal profile was: 95 °C for 5 min, and 40 cycles of 94 °C for 30 s, 45 °C for 30 s, 72 °C for 30 s, and final extension of 5 min at 72 °C.

PCR conditions for TS-16 and TS-18 primers were the same as for the GP primers, except that for TS-16 and TS-18 the annealing temperatures were 61 °C and 63 °C, respectively.

For PC03/PC04 primers, the total 25 μL PCR reaction mixture contained 5 μL sample, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM MgCl₂, 200 μM dNTPs, 0.2 pmol of each primer, and 0.2 U of Taq polymerase. The PCR thermal profile was 94 °C for 5 min, and 40 cycles of 94 °C for 30 s, 51 °C for 30 s, 72 °C for 30 s, and 5 min final extension at 72 °C.

Table 1 Primers used for HPV typing

Primer	Sequence	Target gene	Amplimer length
GP5	TTTGTTACTGTGGTAGATAC	L1	155 bp
GP6	GAAAAATAAACTGTAAATCA		
TS16-A	GGTCGGTGGACCGGTCGATG	L1	96 bp
TS16-B	GCAATGTAGGTGTATCTCCA		
TS18-A	CCTTGGACGTAAATTTTTGG	L1	115 bp
TS18-B	CACGCACACGCTTGGCAGGT		
PC03	ACACAACGTGTTCACTAGC	β -Globin	110 bp
PC04	CAACTTCATCCACGTTCCAC		

References for each primer are cited in the Materials and methods section. GP, general primer for L1 HPV; TS, type-specific primers for L1 HPV; PC0, primers for β -globin gene.

Amplified PCR product was run on 2% agarose gel and stained with ethidium bromide. The PCR products were identified on the basis of their predicted fragment size.

To authenticate our results, several controls were used in the study: (a) To validate the PCR data, DNA from positive control tissues, i.e., cervical carcinoma samples known to be positive for HPV subtype 16 or 18, was subjected to PCR. (b) To confirm successful DNA extraction, PC03/PC04 primers were used to amplify β -globin gene. (c) Negative PCR controls were included without DNA template to control for template contamination. (d) To control for DNA contamination during sample preparation, HPV-negative control tissue was processed on the microtome along with the cervical carcinoma samples. The entire batch of these samples, including the negative control tissue, was subjected to DNA extraction and then to PCR in a single session.

Results

We estimated the prevalence of HPV in 60 cervical carcinoma biopsies from Pakistani patients coming from diverse cultural, social, and economic strata. Our results showed 59 of the samples to be positive for HPV. The subtype found in 57 of the 60 samples was HPV16, whereas HPV18 was detected in only one sample.

Discussion

The study reported here is the first of its kind to gain an understanding of the prevalence of HPV in Pakistani patients with cervical carcinoma. The work was performed retrospectively, using paraffin-embedded cervical carcinoma biopsies taken from 1991 to 2005, from two different hospitals in Karachi. The hospitals approached for this study, Aga Khan University Hospital and Civil Hospital, are two of the major healthcare centers in Pakistan, where patients from all walks of life are received for treatment. To ensure that the patients for this study were chosen from a diverse background, the only selection criteria used for the patients was gender (female) and cancer diagnosis (positive). In other words, no distinction was made on the basis of ethnic, social, or economic background. Furthermore, no distinction was made between the types of cervical carcinoma either; squamous as well as adenocarcinomas were included in this study.

All samples were screened for HPV using general (subtype-independent) primers, as well as specific primers for the

subtypes 16 and 18. Except for one sample, all 60 samples examined were found positive for HPV. To control against artifacts in the PCR protocol, several controls were included in the experiments. We used a positive and a negative control for each of the HPV subtypes 16 and 18 to exclude the possibility of false negative results. Furthermore, a known HPV-negative paraffin-embedded tissue was processed on the microtome along with the rest of the samples. This negative control was treated in one batch with the rest of the samples for DNA extraction, and then analyzed by PCR in a single session. No PCR products were amplified in the negative control, using either general or specific primers, although a positive band was obtained with β -globin primers, confirming efficient DNA extraction, and excluding contamination-generated artifacts (Figure 1).

Worldwide, HPV prevalence in cervical carcinoma is reported to be 99.7%.¹² Prevalence of HPV in Pakistani

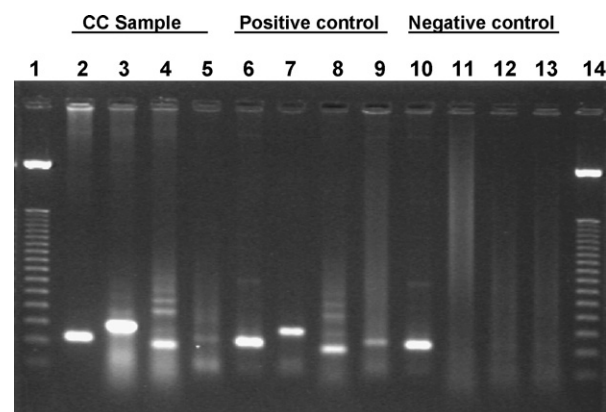


Figure 1 Representative gel of HPV PCR. Lanes 1–14 show the 50 bp molecular weight ladder. Lanes 2–5 are results from a cervical carcinoma (CC) specimen. PCR primers used: β -globin, lane 2; HPV (general primers), lane 3; HPV subtype 16, lane 4; HPV subtype 18, lane 5. Lanes 6–9 are positive controls for HPV, HPV16, and HPV18; primer order is the same as for lanes 2–5. Lanes 10–13 are negative controls for HPV, HPV16, and HPV18; primer order is the same as for lanes 2–5. Negative and positive controls are described in the Materials and methods section. The cervical carcinoma specimen is positive for β -globin, HPV, and HPV16, but negative for HPV18. The positive control has expected amplimers in all four lanes. The negative control shows no amplification by HPV, HPV16, or HPV18 primers; a positive band is observed, however, for β -globin gene.

cervical carcinoma patients was found to be similar; 59 out of 60 cervical carcinoma specimens were found positive for HPV. Ubiquity of HPV subtype 16 in cervical carcinoma may reflect a genetic and racial susceptibility of Pakistani women to this particular subtype. In India, too, a high prevalence of HPV16 has been reported among women.¹³ Since the Pakistani population shares its ancestral roots with India, it is possible that the similarity in genetic makeup has translated into a similar host response towards HPV infection. It is very likely a genetically-determined, host-driven phenomenon that favors propagation of subtype 16 in the Pakistani population.

Our results underscore the importance of screening for STIs in the asymptomatic Pakistani population. Although reports in the past have suggested that Muslim women are less susceptible to HPV infection,¹⁴ a recent study from India has shown no difference between levels of susceptibility for HPV among Muslim and non-Muslim women.¹⁵ Our study supports the latter observation. Historically, screening for STIs has never been considered important in Muslim countries, under the assumption that sexual promiscuity, and therefore sexually transmitted disease, in a Muslim population is nonexistent. The gravity of this matter is further aggravated by the socio-cultural taboos that prohibit investigation of all matters pertaining to sex and STIs. In certain countries including Pakistan, where religious and cultural values strictly discourage sexual promiscuity, protection from sexually transmitted infections, such as HPV, is taken for granted. It is for this reason that on a national level, the need to screen for STIs and increase public awareness about the same has never been identified. Consequently, hospitals and other healthcare facilities in Pakistan do not routinely prescribe HPV screening to female patients. It is not common practice to perform Pap smears on Pakistani women. As a result, the frequency of HPV occurrence in the female Pakistani population is virtually unknown.

The results of our study indicate that sexual non-promiscuity in Pakistani society could well be a myth. Although HPV is not always spread through sexual intercourse,¹⁶ it does appear to be the predominant mode of transmission. The fact that nearly all cervical carcinoma patients were found positive for HPV could mean that either the patients or their partners had engaged in unsafe and/or extramarital sexual activity. In that case, there is a possibility of other STIs being present as well. This calls for efforts to be invested into improving awareness among men and women, particularly those socio-economically disadvantaged, about safe sexual practices that decrease the risk of contracting STIs. Reducing the overall occurrence of STIs could potentially decrease HPV infection and, consequently, the incidence of cervical carcinoma.

The socio-cultural taboos on sex become a major barrier in establishing the epidemiology of HPV in Pakistan. Due to their lack of awareness, people do not get screened for STIs. Due to the stigma attached to sexual diseases, people also choose not have their STI status diagnosed. Our results highlight the need for regular cervical carcinoma screening in asymptomatic Pakistani adult females. To date, cervical carcinoma screening tools used in research on Pakistani women include Pap smears to look for dyskaryosis and visual inspection of the cervix using acetic acid.¹⁷ Since

our study has confirmed a strong association of HPV with cervical carcinoma, HPV typing can be used as a very effective screening tool for cervical carcinoma in Pakistan. Early detection of infection with a high-risk subtype could give the patient an opportunity for more effective management of the disease, as early treatment of HPV infection has been shown to reduce the progression to malignancy.¹⁸

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Conflict of interest: No conflict of interest to declare.

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