Molecular Detection and genotyping of human papilloma virus in cervical specimens among Egyptian Female Patients

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Abstract: Human Papilloma Virus (HPV) was found to be involved in a variety of malignancies; cervical cancer is the most important and prevalent. The goal of this study was to identify genital HPV: to determine its rates and possible genotypes in cervical biopsies from Egyptian female patients; to detect its association with non malignant and malignant cervical lesions and to examine the potential role of HPV in development of cancer cervix. This study was carried out on 60 Egyptian female patients with histopathological evidence of flat condyloma, CIN and cervical carcinoma in addition to 30 age matched females as control group. The molecular analysis was carried out employing MY09/11 consensus HPV L1 PCR in order to molecularly detect genital HPV. Positive PCR samples for HPV were further subjected to molecular genotyping by Southern blot using specific labeled oligonucleotide probes **(6-11-16-18)** followed by sequencing for confirmation. PCR detected HPV DNA in 76.7% of patients and in 10% of the control group. *The HPV was positive in 84.2% of patients with flat condyloma lesion of cervix; in* 71.4% of the CIN group and in **75%** of cancer cervix patients. By Southern blot genotyping, it was found that in flat condyloma HPV genotype 6 was in 62.5% followed by genotype 11 (18.8%). CIN lesions harbored high risk oncogenic HPV genotypes in 53.8%. As regards squamous cell carcinoma HPV genotype 16 was found in 90.9% while HPV 18 was the only genotype detected in adenocarcinoma. In conclusion, HPV infection was found to be common and more associated with CIN II & III lesions and invasive carcinomas. This reflects a large unscreened population so introduction of newer techniques in female screening should be a matter of intense research. HPV DNA detection and genotyping is useful for classifying oncogenic HPV thus serving as a valuable tool in picking up of high risk group.
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1. Introduction

Human papillomavirus (HPV) is a nonenveloped, double-stranded DNA virus. Most infections clear within 2 years, however, a minority persists and potentially could progress to cervical cancer (1). HPV have more than 100 genotypes (2), infection with certain genotypes, that are high-risk HPV (16&18), play an essential role in the development of uterine cervical cancer (3)**.** In contrast, other genotypes, as HPV 6 and 11, are considered as 'low risk' for the development of cervical carcinoma (4).

In malignant transformation of uterine cervical epithelia, viral DNA integrate into the host genome causing disruption of the HPV 16 E2 gene -a negative regulator of the E6/E7 promoter. Consequently, increasing expression of E6 and E7 viral oncoproteins that target the p53 and retinoblastoma (pRb) tumor suppressor proteins, respectively, thus down regulating their antitumor functions (5).

Since HPV cannot be cultured and the clinical performance of serological assays is poor, diagnosis of HPV infection is almost entirely based on molecular tools (6), including liquid hybridization as Hybrid Capture 2 (7), Southern and dot blot hybridization using HPV type-specific probes, type-specific polymerase chain reaction (PCR) (6), and general-primer PCR (8,9)

Polymerase chain reaction (PCR) primers that target the highly conserved regions of the HPV L1 open-reading frame are capable of amplifying a broad spectrum of HPV types (10) and the newly developed short-fragment PCR (11).

Accordingly, the aim of this study was to identify genital HPV; to determine its rates and possible genotypes in cervical biopsies from Egyptian female patients; to detect its association with non malignant and malignant cervical lesions and to examine the potential role of HPV in development of cancer cervix.

2. Subjects and Methods:

1-Study population:

After informed consent, 90 women attending the Early Cancer Detection Unit, Department of Obstetrics and Gynecology, Ain-Shams University Hospitals were participated in this study, in the period from June 2008 to May 2010. Their ages ranged between 28-52 years with a mean age of 39.5 ± 11.4 years. According to the histopathological results the subjects studied were classified into:

Patient group (I): comprising 60 patients.

Ia: 19 patients with flat condyloma lesion of cervix.

Ib: 21 patients with Cervical Intraepithelial Neoplasia CIN (I, II, III).

Ic: 20 patients with cancer cervix; 16 patients with squamous cell carcinoma (SCC) and 4 patients with adenocarcinoma (AD).

In addition to 30 females with normal histological findings were considered as control group (II).

Subject enrollment criteria included: no history of low or high-grade cervical squamous intraepithelial lesions within the past year (i.e., not attending clinic for referral abnormal Pap smear), not currently pregnant and no hysterectomy.

All studied groups were subjected to:

-Through detailed history laying stress on age, ethnicity, marital status, household income, education level, age at menarche, parity, smoking history, history of use of condoms, diaphragm, and birth control pills, history of partner with genital warts, history of sexually transmitted disease (STD) (chlamydia, trichomonas, gonorrhea, and herpes), and history of pelvic inflammatory disease.

-Routine gynaecological examination and cytological evaluation, colposcopic examination with directed punch biopsy and histopathological evaluation.

-HPV-DNA detection using the MY09/11 consensus HPV L1 PCR.

-Southern blot hybridization for HPV genotyping followed by HPV-DNA sequencing for positive samples for confirmation of genetic specificity.

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2. Methods:

2.1 Cytological evaluation (Pap test):

Cervical cell scrapings were collected with a cytobrush from the ecto- and endo-cervix. The cytobrush was rolled onto 2 separate glass slides which were then fixed in 95% alcohol for Pap test. The microscopic cellular changes in the cervicovaginal smear were detected according to Bethesda classification system (12). All women with abnormal Pap smears were referred to the colposcopy.The main diagnostic features of flat condyloma is Koilocytes showing a distinct perinuclear zone of cytoplasmic clearing together with peripheral zone of dense cytoplasm and atypical nucleus. Also dyskeratocytes (individual cell kertinization: parakeratotic and hyperkeratotic cells) are commonly found however not specific for HPV (13).

2.2 Colposcopic examination

Together with saline test, acetoacetic test and Schiller test for all patients with abnormal Pap smears (14). Colposcopic directed punch biopsy from the abnormal part of the cervix detected by acetoacetic acid test and Schiller test. Each biopsy was divided into 2 parts. One part was kept in formalin for histopathological examination, the other part was immediately stored at -70ºC in aluminum foil for PCR detection of HPV and Genotyping

2.3 Histopathological evaluation:

Sections of biopsies were prepared, stained with hematoxylin-eosin and examined by a single pathologist. Tissues biopsies were classified as either normal cervix (including cases of chronic inflammation), flat condyloma, CIN (I , II or III) and cancer cervix either squamous cell carcinoma, or adenocarcinoma. The pathologist had no clinical information or results of cytology, colposcopy or HPV testing (Fig1-3).

2.4 HPV detection using the MY09/11 consensus HPV L1 PCR (15):

Total DNA was extracted from approximately 25 mg of tissue biopsy using the DNeasy tissue kit (Qiagen Inc., California, USA), following the manufacturer's protocol. The polymerase chain reaction (PCR) targeting the L1 capsid protein gene was used to amplify a 458-base pair DNA fragment using consensus primers the MY09/11 that amplify a wide range of HPV genotypes. Reactions were performed in 50 ul volumes containing 0.5 mg of DNA, 200 pmol of each primer, 0.2mM of each dNTPs, 1unit thermostable Taq DNA polymerase and 1X Reaction buffer (all reagents were supplied by Promega, USA).

Cycling conditions were performed as follows: one initial denaturation step at 94 ºC for 1 min; 40 cycles of denaturation at 94º C for 1 min, annealing at 50 ºC for 1 min, and extension at 72ºC for 2 min; and one final extension step at 72 ºC for 10 min. Amplified DNA fragments were resolved by electrophoresis of 40 ul of PCR products in 2% agarose containing ethidium bromide (0.5 mg/ml) and visualized under UV transilluminator. The expected PCR products size was 458 bp (Fig4).

Each experiment was performed with separate positive and negative PCR controls. Amplification of isolated DNA was checked by β-globin PCR primers PC03 and PC04, generating a fragment of 110 bp [20].

2.5 Southern blot hybridization for HPV genotyping:

The oligonucleotides type specific probes for types 6,11,16 &18 were as follows:

Type 6: 5' CAT CCG TAA CTA CAT CTT CCA 3'

Type 11: 5'TCT GTC TCT AAA ACT GCT ACA 3'

Type 16 : 5' CAT TAC ACT CCA GCA CCC TGA 3'

Type 18: 5' GGA TGC ACC GGC TGA 3'. They were labeled using DIG oligo nucleotide 3' End Labeling Kit supplied by Boehringer Mannheim.

For typing by southern blot, electrophoresis was repeated for all positive HPV-PCR amplified products to collect them on separate membrane**.** DNA from agarose gel was then transferred by capillary blotting to the nylon membrane (Amersham, UK) using 0.4 M NaOH buffer, then the membrane was hypridized with HPV 6,11,16&18 Digoxigenin labeled oligonucleotides probes. Then the membrane was exposed to X-ray film resulting in an autoradiograph (16). To denote the genotype of HPV, the resulting bands were compared to corresponding sites of specific bands originally present in gel (Fig 5).

2.6 DNA sequencing:

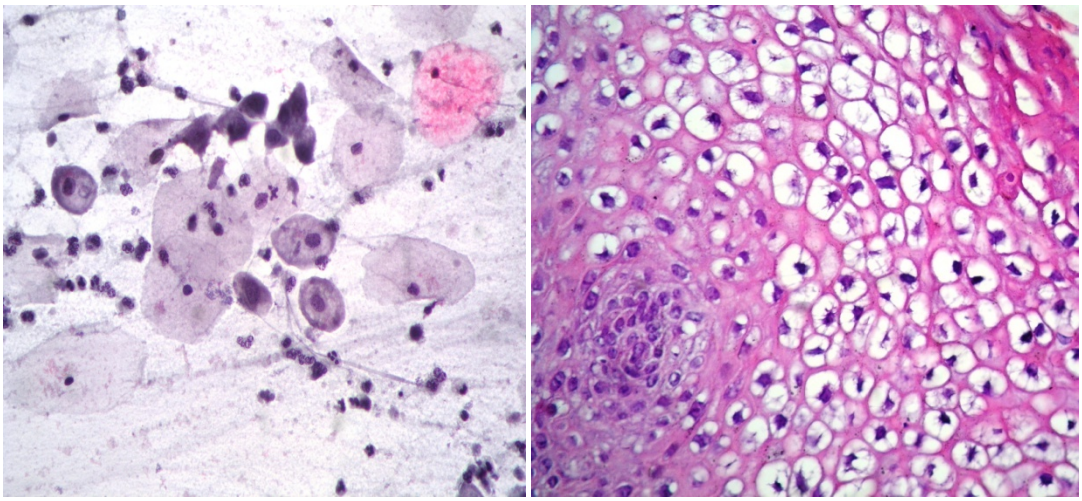
A selection of HPV DNA positive samples was sequenced for confirmation of genetic specificity. The sequencing was performed with a dye terminator cycle sequencing kit (Applied Biosystems,UK). The sequences were analyzed on an ABI PRISM 310 genetic analyzer, Perkin Elmer (Applied Biosystems, UK) according to their protocol.

The sequences were compared to other known sequences in a database search (BLAST from National Center for Biotechnology Information [NCBI]: http://www.ncbi.nih.gov/)

Statistical analysis:

The association between the detection of HPV DNA and its specific types were described by using chi-square (X^2) test. While relation to parametric data, as age

and duration of marriage, were described by Student t-test and were computed by SPSS procedures.



A B

Fig. 1: A- cervicovaginal smear; flat condyloma Pap stain X100 B- Flat condyloma of the cervix (LGSIL) H&E stain X400

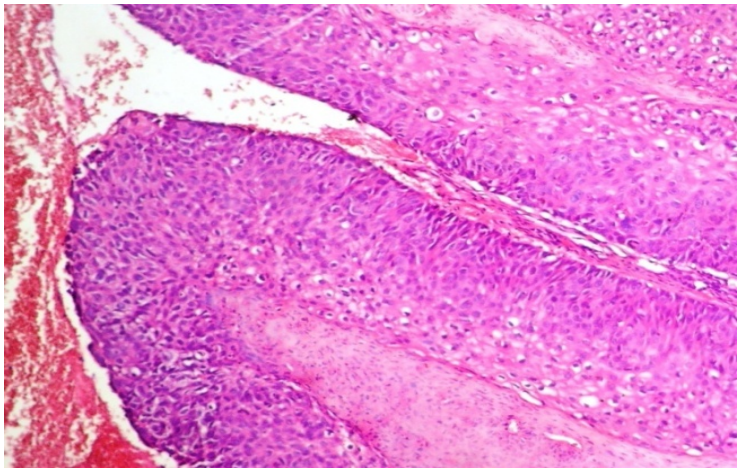


Fig. 2: Carcinoma insitu (CIN III) H&E stain X400

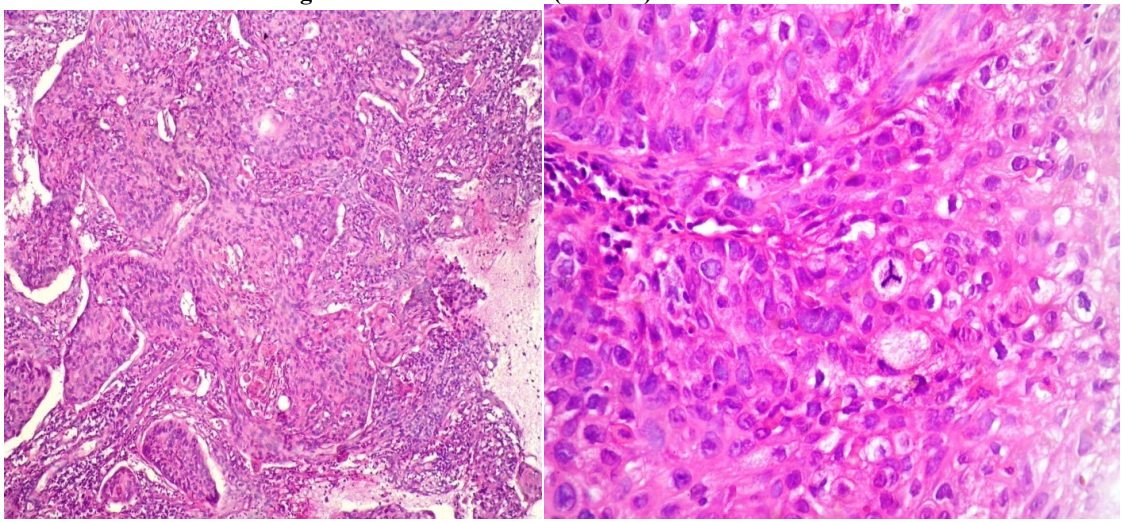


Fig. 3: squamous cell carcinoma of the cervix grade II, H&E stain X 100& X400

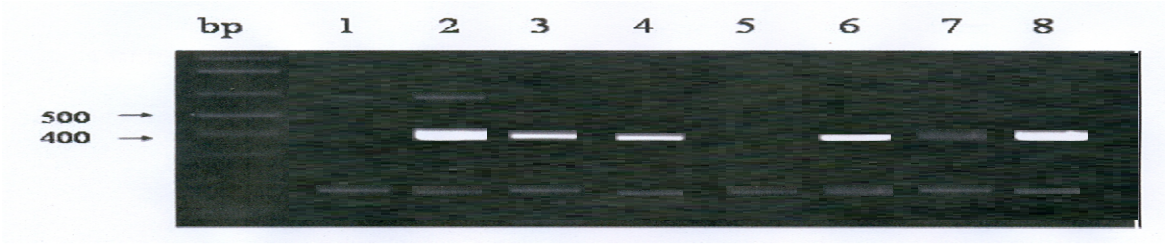
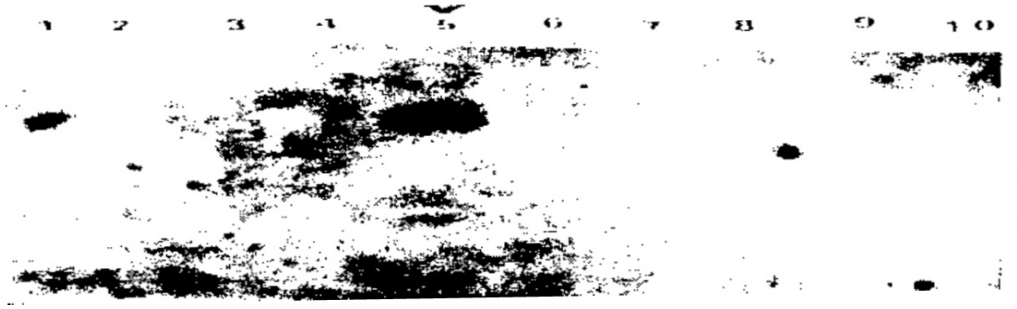


Fig. 4: Agarose gel electrophoresis of amplified DNA fragments corresponding to the L1 capsid protein gene.

Probe 6

Probe 11



Probe 16

Fig. 5: Southern blot autoradiograph showing hyperidization of HPV DNA

3. Results:

Cytological findings:

The control group (30) reveals no cellular abnormalities in all cases. In the study group (60) cytologic diagnosis was low-grade squamous intraepithelial lesions (LGSIL) in 24 and high-grade squamous intraepithelial lesions (HGSIL) in 36 of cases.

Histopathological findings:

No significant histopathological changes were seen in the control group (30). In the study group, histopathology confirmed the diagnosis of LGSIL in all 24 cases which classified as 15 flat condyloma & 9 CINI. While in HGSIL cases, histopathology proved the diagnosis in 30 out 36 case as following: 9 CINII, 1 CINIII, 20 carcinoma (16 SCC& 4 AD). The remaining 6 cases were diagnosed as LGSIL (4 flat condyloma, & 2 CINI)

HPV DNA analysis:

HPV DNA was detected in 76.7% of patients while it was detected in 10% of the control group. Age and duration of marriage showed no significant difference between HPV-DNA positive and negative cases (t=0.08&0.35 respectively; p>0.05).

HPV DNA was detected in 84.2% of patients with flat condyloma lesion of cervix. In CIN group HPV DNA was positive in 15/21 (71.4%) [CINI: 7/11 (63.6 %), CIN II: 7/ 9 (77.8%)] and the only case of CIN III was positive. Among the group with cancer cervix, HPV-DNA was detected in 15/20 (75%); squamous cell carcinoma were positive in 11/16 (68.8%), while all adenocarcinoma cases were positive for the HPV DNA (4/4) (Table 1).

HPV gynotyping

The genotype distribution among different groups is shown in table 1.

DNA sequences of high-risk HPV types by used probes (HPV16&18) were detected in 23/43 (53.5%) of patients groups while low-risk HPV types (HPV6&11) were present in 20/43 (46.5%). The remaining 3 untyped cases by the used probes were excluded (Table2).

All flat condyloma cases carried low risk types except for a case (6.2%) carrying the high risk type (HPV 16). Among the CIN group, 7/13 (53.8%) of the positive HPV DNA cases carried high risk types [CINI: 2/6 (33.3%), CINII: 4/6 (66.7%), CINIII: 1/1 (100%)]. While all cancer cervix group carried high risk genotypes.

Majority of squamous cell carcinoma (90.9%) had a single infection with HPV type 16, while adenocarcinoma cases showed infection with HPV type 18 only.

Three mixed infections were detected among 3 cases; one case of flat condyloma carrying HPV 6&11; one case with CINI showing 6&11 types and one case of SCC revealed types (16&18). The distribution of HPV genotypes (high or low-risk) among different patients groups showed a high statistical significant difference (X^2) = 24.6; p=0.0001; Table2).

Sequencing

The genotype of all positive cases for the HPV DNA were further analyzed & confirmed by sequencing. The three untyped case were excluded.

Table (1): HPV-DNA PCR and genotyping in patients and controls

Groups (n)	PCR		HPV-6 (14) n(%)	HPV-11 (7) n(%)	HPV-16 (17) n(%)	HPV-18 (5) n(%)	#Mixed (3) N (%)	•Untyped (3) n(%)
	Positive (49) n (%)	Negative (41) n (%)						
FC(19)	16(84.2)	3(15.8)	10(62.6)	3(18.8)	1(6.2)	-	1**(6.2)***	1(**6.2**)
CIN (21)								
CINI(11)	7(63.6)	4(36.4)	1(14.3)	2(28.6)	2(28.6)	-	1(14.3)*	1(14.3)
CINII(9)	7(77.8)	2(22.2)	1(14.3)	1(14.3)	4(57.1)	-		1(14.3)
CINIII(1)	1(100)	-	-		-	1(100)		-
Ca Cx (20)								
SCC(16)	11(68.7)	5(31.2)	-	-	10(90.9)	-	1(9.1)**	-
Adeno(4)	4(100)	-	-	-	-	4(100)		-
Control (30)	3(10)	27(90)	2(66.7)	1(33.3)	-	-	-	-

FC: flat condyloma; CIN: cervical intraepithelial neoplasia Ca Cx: cancer cervix; SCC: squamous cell carcinoma; Adeno: adenocarcinoma
#Mixed infection: * infection with (6 &11) genotypes ** infection with 16&18 genotypes ●untyped types by used probes were excluded

Table (2): High and low risk-HPV genotypes distribution among patients carrying HPV DNA

Groups (43)* (n)	Low Risk(20) n(%)	High Risk(23) n(%)	X^2	*P*
FC (15)	14(93.3)	1(6.7)		
CIN (13)				
CINI(6)	4(66.7)	2(33.3)		
CINII(6)	2(33.3)	4(66.7)		
CINIII(1)	-	1(100)		
Cancer Cx (15)				
SCC(11)	-	11(100)		
Adeno(4)	-	4(100)	24.6	0.0001

FC: flat condyloma; CIN: cervical intraepithelial neoplasia Ca Cx: cancer cervix; SCC: squamous cell carcinoma; Adeno: adenocarcinoma
*untyped cases by used probes were excluded

4. Discussion

Human Papilloma Virus (HPV) is becoming a menace worldwide, especially to the developing world, due to its involvement in a variety of malignancies, with cervical cancer being the most important and prevalent (17). There are many HPV genotypes; HPV 16&18 are the most carcinogenic. They can cause a variety of low or high-grade cellular abnormalities, most frequently detected in a routine Pap test. On the other hand, low-risk HPV types (LRHPV), as 6 and 11, are mostly associated with benign genital lesions and rarely progress to cancer (18).

Our results confirm the high prevalence of HPV infection among women with genital lesions showing abnormal pathology, as the HPV-DNA was detected in 76.7% of study group. It was positive in 84.2% of flat condyloma; in 63.6 % of CINI; 77.8% of CINII. While, it was detected in the case with CINIII and 75% cancer cervix. This results indicate that the prevalence of HPV infection increased with the severity of cervical lesions (2).This is in agreement with Kroupis & Vourlidis and Castle et.al(6,19) who consider HPV to be the main etiological factor for the development of cervical intraepithelial neoplasia (CIN) and cervical cancer.

HPV-DNA negative cases in this work were 25% of cervical cancers, 28.6% of CIN cases and 15.8% of flat condyloma. These results represent true negative samples for HPV-DNA as the success of amplification process with an internal control β-globin support the integrity of DNA and confirm absence of PCR inhibitors (7).

This study showed that age and duration of marriage have no significant association with HPV infection. On the contrary, other studies showed that age and sexual behavior are key risk factors for HPV infection (10, 17). This discrepancy may be explained by the difference in the study population and cultural behavior.

In the current study, HPV-DNA was detected in 10% of the control. Their molecular genotyping showed low risk types 6&11. These results strongly suggest that these cases represent subclinical or latent infection. This is in agreement with a previous studies who stated different rates of HPV detection in normal cervical samples (14,20,21). In cases with normal cervical cytology, HPVDNA was detected in a wide range from 3 to 34.3% (6,10) . This wide range is explained by the different nature of participating populations in such studies, and by technical evolutions in the diagnostic tests used (11). Others added that these women should be considered as having a real risk for progression to abnormal cytological findings (22).

HPV genotypes are classified according to their neoplastic ability as high-risk and low-risk types. High-risk HPV types 16,18,31and 33 are associated with cervical cancer or advanced precancerous stages CIN II and III, which are cytologically characterized as high-grade squamous intraepithelial lesions (HGSIL) (14). On the other hand, low-risk HPV types 6&11 are mostly associated with benign genital lesions such as flat condyloma and CINI, characterized cytologically as low-grade squamous intraepithelial lesions (LGSIL) which rarely progress to cancer (4).

Similarly in this study, the molecular genotyping to HPV-DNA in flat condyloma revealed low risk HPV types in 93.3% of patients while only one case carries high risk type . These results are similar to those reported that the majority of the flat condyloma are associated with HPV-6 and 11 (7). While the case carrying the high risk genotype should be considered as having a real risk for progression to cervical neoplasia (21). Thus, these women should be prospectively followed up by their gynecologist and submitted to appropriate testing cytology, colposcopy and other (23).

In the current report, molecular genotyping revealed that there is high incidence of the high risk HPV genotypes in precancerous CINII and III & cancer cervix. HPV-16 was found to be more predominant in squamous cell carcinoma while HPV-18 was the predominant type in adenocarcinoma. This is in accordance with previous results of Kjaer (24) denoting that different genotypes have different pathologic potentials.

Furthermore, our results are in agreement with a previous study showing that low- risk HPV were seldom detected in invasive cancers (2). On the other hand other reports showed the presence of low risk HPV associated to malignant tumors (25). This could be explained by geographic distribution which is an important variable for the prevalence of a particular type of HPV in CIN and cancer cervix.

In this study, single or multiple infection with high risk oncogenic types (16 or 18) were strongly associated with the diagnosis of invasive carcinoma. This is in concordance with previous publications (3, 23)

In conclusion, HPV infection was found in a large proportion of the population and was more associated with CIN II and III lesions and infiltrating carcinomas. This is indicative of a largely unscreened population thus the effect of introduction of newer techniques in population screening is a matter of intense research. HPV DNA detection and genptyping method could be useful for classifying oncogenic HPV and serve as a valuable tool in monitoring of HPV-related disease with a higher sensitivity, reliability and is relatively inexpensive.

Thus, pointing to the importance for developing preventive protocols and appropriate intervention targets. Furthermore, prophylactic vaccines for HPV 16/18 may be efficient and raise high expectations for the complete eradication of these types in the future.

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