

# Prevalence of Human Papilloma Virus Infection in Malignant Lesions of the Uterine Cervix in Morocco.

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## Abstract

*Cervical cancer is one of the most common tumors afflicting women with a disproportionate mortality occurring in developing countries. The aim of this study was to determine the prevalence of high-risk oncogenic human Papilloma viruses (HPVs) in 193 archival cervical lesions of Moroccan women. The presence of high-risk HPVs in paraffin-embedded, formaldehyde-fixed cervical lesion specimens was detected by Polymerase Chain Reaction (PCR) using consensus primers complementary to late 1 (L1) gene of the genital HPVs. Amplified PCR products were verified and typed by Southern blot analysis using P-32 labeled DNA probes. HPV prevalence was found to be 89% in malignant lesions (138/155) and 68.4% in premalignant lesions (26/38). HPV 16 was detected in 61% (100/164); whereas HPV 18 in 33.5% (55/164), HPV 31 in 1.8% (3/164), HPV 33 in 1.2% (2/164), HPV 35 in 7.3% (12/164), and HPV 45 in 3.7 % (6/164) of lesions. Notably, multiple HPV type co-infections occurred only in malignant lesions and were present in 20.1% (33/164) of cases. The most common co-infection was HPV types 16 and 18, followed by HPV types 16 and 35. Thus, a high prevalence of HPV16/18 was observed in archival cervical cancers. Hence, relevant HPV typing information in cervical cancer is very important for further HPV vaccines design and application.*

*Key Words: HPV, cervical cancer, prevalence, Morocco*

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## Introduction

Worldwide cervical cancer is the second most common malignancy in women with nearly a half million new cases diagnosed and 200,000 deaths each year (1). In Morocco, cervical cancer represents a serious public health problem, and in the absence of a national cancer registry, data are limited to the number of cases registered in some medical centers. The hospital-based cancer registry of the National Institute of Oncology (INO), Rabat, reports more than 500 new cases annually (2).

Cervical cancer is considered a sexually transmitted disease (3). Clinical and epidemiological studies have identified the human Papilloma virus (HPV) as the central risk factor for cervical cancer development (4). HPV are strictly epitheliotropic viruses infecting cutaneous or mucosal surfaces (3). To date, more than 200 HPV genotypes have been identified (5), but the interest is focused only on genital HPV (40 genotypes) that are associated to precancerous and cancerous lesions of the cervix (6). HPV genotypes vary in oncogenic potential and are associated with anatomically and histologically different diseases. They have been grouped into low risk and high risk types based on the frequency of association with invasive cervical cancer. HPV 16 and 18 genotypes, which are frequently found in association with cervical cancer, are considered as the most oncogenic types. Others HPVs, 31, 33, 35, 45, 51, 52, 58 and 59, that are also considered as carcinogenic are less frequent in cervical carcinomas (7, 8, 9). When HPV infection occurs, the viral DNA is present in the cell as an episomal plasmid and the infection occurs in the majority of cases due to host immune response. Persistence of infection favors viral integration in the cell genome which, together with other factors, can progress to high-grade squamous intraepithelial lesion (HSIL) and cancer (10,11).

Usually, there is a long latency period between the time of HPV infection and the cancer appearance. In this study, we report results of molecular detection and typing of oncogenic HPV genotypes in 193 cervical lesions using PCR combined with probe analysis.

## Materials and Methods

All 193 uterine cervix samples, studied, were all collected from the "Institut National d'Oncologie" (INO), Rabat, and from the Oncology Department of "Centre Hospitalier

	N	HPV+ Cases	HPV 16	HPV 18	HPV 16+18	HPV 31	HPV 33	HPV 35	HPV 45	HPV 16+35	HPV 16 + 18+45	HPV 18 +35	Undetermined Types
<i>in situ</i> carcinoma Epidermoid Carcinoma	4	3	0	0	0	0	0	0	1	0	0	0	2
Adenocarcinoma	141	129	56	24	18	3	1	2	1	8	4	2	10
Sarcoma	8	5	1	1	1	0	0	0	0	0	0	0	2
Total	2	1	1	0	0	0	0	0	0	0	0	0	0
<b>Total</b>	<b>155</b>	<b>138</b>	<b>58</b>	<b>25</b>	<b>19</b>	<b>3</b>	<b>1</b>	<b>2</b>	<b>2</b>	<b>8</b>	<b>4</b>	<b>2</b>	<b>14</b>

**Table 1.** Distribution of HPV infection in malignant lesions.

	N	HPV+ Cases	HPV 16	HPV 18	HPV 33	Undetermined Types
NSI	30	22	9	5	1	7
Condyloma	3	1	1	0	0	0
CIN	2	1	1	0	0	0
Epidermoid Metaplasia	3	2	0	0	0	2
<b>Total</b>	<b>38</b>	<b>26</b>	<b>11</b>	<b>5</b>	<b>1</b>	<b>9</b>

**Table 2.** Distribution of HPV infection in precancerous lesions

Universitaire Ibn Rochd” (COIR), Casablanca. Specimens were fixed in 10% buffered-formaldehyde, paraffin-embedded, sectioned at 5 µm, and stained with hematoxylin-eosin (12, 13). Diagnosis was made by a pathologist.

DNA was extracted from paraffin-embedded, formaldehyde-fixed tissue blocks. Sample sections (3 to 5 section of 5 µm for each sample) were deparaffinated with xylene, and rinsed with ethanol to remove the remaining xylene. Cells were then lysed in the digestion buffer (Tris-HCl 10 mM pH 8.0, EDTA 10 mM, NaCl 150 mM and SDS 2%) containing proteinase K (0.1mg/ml). DNA isolation was performed with phenol-chloroform extraction and ethanol precipitation. DNA was then resuspended in sterile distilled water, and stored at -20°C until use (14).

The first aliquot of a given extract was screened with a β-globin PCR using PC03 and GH20 primers that allow the amplification of a 123 bp fragment (15). This first amplification is used to check the DNA quality and competence. A second aliquot was used for HPV DNA detection and typing. DNA was amplified by the polymerase chain reaction (PCR) using consensus primers: MY 09 and MY 11 (16). Amplification reaction was performed in total volume of 50 µl. The amplification mixture contained 50 pmol of each consensus primer, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.625 units *Taq* DNA polymerase (purchased from Amersham) and 5 µl of DNA sample in 1x *Taq* polymerase buffer. The mixture was first denatured at 94°C for 7 min. Then, thirty-five cycles of PCR were performed with denaturation at 94°C for 30 s, primer annealing for 1 min at 56°C and primer extension for 1 min 30 s at 72°C. At the end of the last cycle, the mixture was incubated at 72°C for 7 min. For every reaction, a negative control in which DNA template was omitted from the amplification mixture, and a positive control are included.

**Hybridization analysis of PCR products**

Aliquots of 10 µl of the PCR product were analysed by electrophoresis through 1% agarose gel. The 50 bp ladder molecular-weight marker (Amersham) was included for

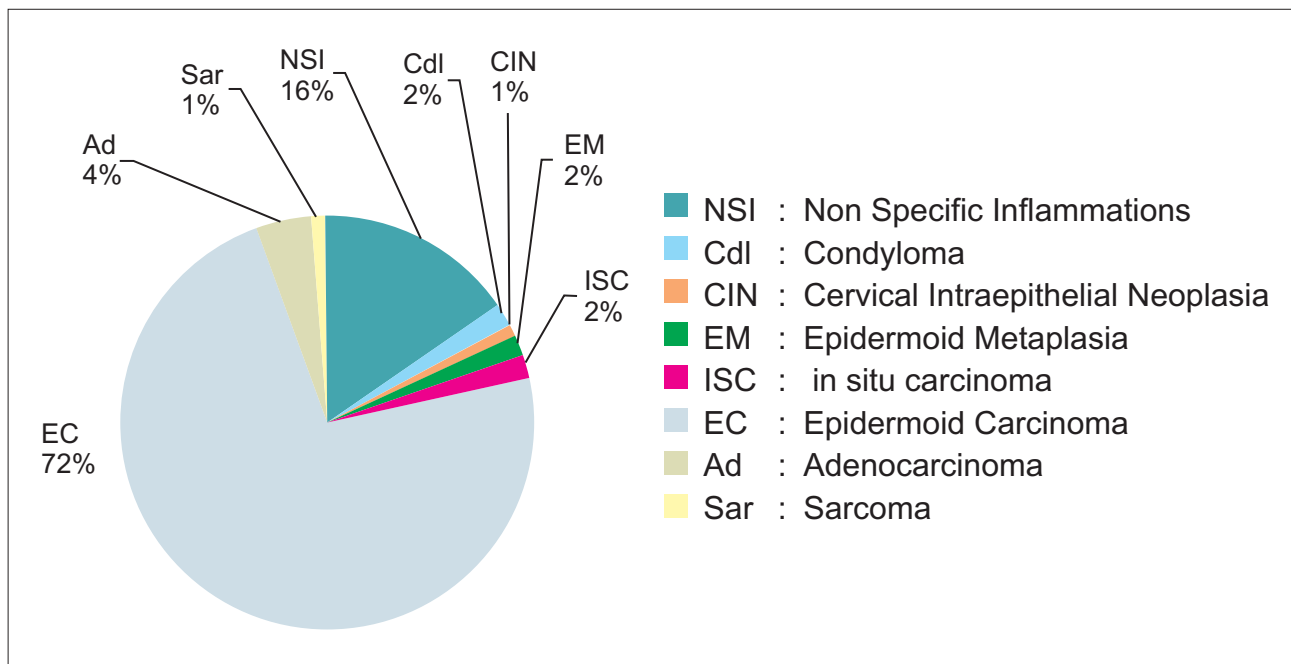
detection of the DNA size of the amplification product. PCR products were transferred onto positively charged nitrocellulose membranes (Hybond N+, Amersham) and fixed by incubation at 80°C for 2h. HPV specific probes were end-labeled by phosphorylation with [γ-32P]-ATP (Amersham) and Hybridized under stringent conditions to amplicons on the membrane as described previously (14). The probes used in this study were MY14 (C A T A C A C C T C C A G C A C C T A A), WD74 (G G A T G C T G C A C C G G C T G A), WD126 (C A A A G C C C A A G G A A G A T C), MY16 (C A C A C A A G T A A C T A G T G A C A G), MY115 (C T G C T G T G T C T T C T A G T G A C A G) and MY70 (T A G T G G A C A C T A C C C G C A G) that are specific to HPV 16, 18, 31, 33, 35 and 45 respectively (16). Membranes were then washed under normal and stringent conditions and specific bands were revealed by autoradiography.

**Results**

The pathological results of the 193 biopsies are represented in the Figure 1. Two types of lesions prevail, malignant lesions with 80.3 % of cases (155/193), and lesions diagnosed as non-specific inflammations (NSI) with 15.5 % of cases (30/193). Malignant lesions included epidermoid carcinoma, adenocarcinoma, *in situ* carcinoma, and sarcoma.

The presence of amplifiable DNA, using primers for a fragment of β-globin gene, was confirmed in all cases and all DNA samples were adequate for further analysis. The MY09 and MY11 are consensus primers that allow the amplification of 450-bp target sequences of the L1 region that's highly conserved on a broad spectrum of HPVs. Successful amplification of HPV positive DNA preparations with MY11/MY09 primers yielded DNA fragments of 450 pb corresponding to the PCR products of HPV L1 gene.

Using PCR amplification combined with molecular probing, we revealed the presence of HPV DNA in 85% of cases (164/193) with a predominance of HPV 16, which



**Figure 1.** Histopathological profile of the 193 cases.

was present in 61% of cases (100/164), and HPV 18 in 33.5% of cases (55/164).

The distributions of viral genotypes in the 164, HPV positive, malignant and premalignant lesions are reported respectively in Table 1 and Table 2. A total of 89% of malignant cases were found to be HPV positive (138/155). Molecular typing showed single and multiple infections. HPV 16 and 18, alone or in co-infections, were presents in 64.5% (89/138) and 36.2% (50/138) respectively, whereas the others types gave a low prevalence. Among the multiple infection cases the most common co-infections were HPV types 16/18 (19 cases), followed by HPV types 16/35 (8 cases) and HPV types 18/35 (2 cases). Triple infection with HPV 16/18/45 was also found in 4 cases.

In the NSI group, 68.4% of cases were HPV positive (26/38). Molecular analysis revealed no multiple infections. HPV 16, the most predominant genotype, was present in 42.3% (11/26). HPV 18 and 33 were present in 19.2% (5/26) and 3.8 % (1/26) respectively. No specimen harbored the other genotypes, HPV 31, 35 and 45.

## Discussion

More than 490 000 new cases of cervical cancer occur among women worldwide each year. Incidence rates of this disease vary from about 5 cases per 100 000 women per year in many industrialized countries to more than 50 per 100 000 in some developing nations (17). Approximately 80% of all cases occur in less-developed countries, because prevention programs are either non-existent or poorly conducted (18). Furthermore, in the less developed areas of the world cervical cancer begins to strike significantly among women as young as 25-30 years of age, clearly identifying this disease as the cancer priority in women. In Morocco, cervical cancer is the second most common female cancer after breast cancer and represents a major public health problem. In fact, in a hospital series, cervical cancer appears as one of the most frequent cancers among

women. The diagnosis is usually made in advanced stages, and mortality is high. A statistical study conducted at the INO, between 1985 and 1994 showed that among 4268 cases of cervical cancer, only 5% were in stages I and II, whereas the majority were in advanced stage (2, 19, 20). As an oncology treatment centre, patients come to INO via other local and regional centres, which may explain the higher rate of advanced stages of cancer and especially, cervical cancer, recorded at the INO. We sought, in this study, to determine the presence of HPV in cervical carcinoma from Moroccan women. Molecular techniques, using PCR and specific hybridization, were used to detect HPV DNA in 193 formalin-fixed tissues. DNA amplification was performed using HPV L1 consensus sequence primers (MY11 and MY09) which recognize a broad spectrum of HPV types including HPV type 6, 11, 16, 18, 31, 33, 35, 38, 40, 42, 45, 51–55, and 57–59, as well as many untyped HPVs.

The prevalence of HPV DNA by PCR with the MY09/MY11 primers was 85 % and is in concordance with the overall world distribution of HPV types in cervical cancer (23).

Molecular typing showed that, HPV16 was the most prevalent type in cervical cancer, followed by HPV 18, suggesting that there are the most prevalent genotypes in Morocco, whereas the HPV 31, 33, 35 and 45 were detected in less than 20% of cases. The prevalence of these major HPV types in cervical cancer in the present study was similar to that found in a worldwide study conducted by Bosch *et al.*, which showed that the distribution of HPV types varies geographically (24). Except in Indonesia where the ratio of HPV 16 and HPV 18 in were similar, HPV 16 was found to be the predominant type in all countries studied thus far (24, 25, 26).

Previous reports have shown that HPV 16 is the most prevalent type in epidermoid carcinoma, whereas HPV 18 is the most common type in adenocarcinoma and adenosquamous carcinoma (27, 28). In this study, all HPV

18 were located in epidermoid carcinoma and only one case was in adenocarcinoma. Interestingly, we found that multiple HPV infections were present in about 23.9% (33/138) of our HPV positive cervical cancer specimens, and in the majority of the cases with double infections; HPV 16 was the most detected. Previous studies have shown that multiple infections differ considerably between clinical groups and in relation to the HPV detection assays used. An international survey of invasive cervical carcinoma by the International Biological Study on Cervical Cancer revealed that 4% of specimens harbored double HPV infections (24). Several studies have shown that the incidence of multiple infection was found to be 1.8% in Spain and Colombia (29), 3.7% in Thailand (30), 5.1% in Brazil (31), 9% in the Philippines (32), 12.9% in Peru (33), 19.3% in Paraguay (34) and 32% in Costa Rica (35).

In a previous study it has been reported that infection with multiple HPV genotypes was a factor for persistent HPV infection in healthy young women (36). A recent study also indicated that the presence of multiple HPV infections might contribute to the development or progression of cervical dysplasia (37). Nevertheless, another prospective study found that patients with pre-existing HPV 16 were susceptible to subsequent HPV acquisition, but pre-existing HPV 16 did not affect the subsequent persistence of concomitant infections (38). The complex interaction of concomitant HPV infections in cervical carcinogenesis merits further investigation in the future.

Epidemiologic studies on cervical HPV infections have found that current or prior infection with some HPV types is associated with an increased risk of concurrent or sequential acquisition of other HPV types (39). Although these results are consistent with the hypothesis of synergistic interactions among HPV types, they do not offer conclusive evidence of its validity. Furthermore, no biological mechanism through which current or previous infection with one HPV type predisposes individuals to subsequent infection with another HPV type has been suggested (39). The observed associations may be due to the ensuing physical lesions from HPV infection, analogous to the role played by other sexually transmitted infections, such herpes simplex virus or *Neisseria gonorrhoeae*, in increasing susceptibility to human immunodeficiency virus (HIV) infection (39). Other alternative explanations for this increased risk could be the presence of confounding risk factors or host susceptibility to HPV infection in general. However, it should be noted that all these studies have to be adjusted extensively for confounding variables (e.g., age and number of sexual partners) in the statistical analysis (39).

Current standards for cervical cancer screening in North America and in Europe are based on aggregate detection of the main HPV types presumed to have oncogenic potential, but strong evidence has emerged in recent years in favour of HPV typing as a better prognostic indicator (40). Corroboration of our findings by future studies may assist in the identification of particularly high risk combinations of types present in co-infections, and will likely provide additional impetus for the incorporation of HPV typing in clinical settings. Likewise, as the new era of HPV vaccination begins, improved understanding of the epidemiology of HPV co-infections will also help in

planning HPV testing for the surveillance of immunized individuals (40).

In conclusion, we have showed that HPV 16 and 18 are the most predominant HPV associated with cervical cancer cases in Morocco. HPV testing can play a critical role in the prevention of cervical cancer development. Thus, combination of cytology diagnosis, HPV testing and prophylactic vaccination, especially against these 2 high-risk HPV types, may offer a significant opportunity to the Morocco's National Program against cervical cancer to control this devastating disease and save women's life.

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