CONCISE COMMUNICATION

Human papillomavirus DNA in cervical lesions from Morocco and its implications for cancer control

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To determine the types of human papillomaviruses (HPV) in northern Morocco, information which is needed for the design and use of HPV vaccines, we have analysed 129 cervical biopsies from this region. In our study, 91 cases were HPV positive, 45 cases had HPV-16 DNA, and 20 cases had HPV-18 DNA. This distribution of virus type was similar in inflammatory cervical lesions and in invasive cervical carcinomas. In conclusion, the HPV type distribution in Morocco is similar to that in other African Mediterranean countries, where the proportion of HPV-18 cases is significantly higher than in Europe. Determination of virus-type distribution is essential for vaccination programs.

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INTRODUCTION

Clinical and epidemiological studies have shown that human papillomaviruses (HPV) play a major role in the development of different types of cervical lesions, and are therefore considered as the major infectious aetiological agents of genital lesions and cancers [1]. HPV are strictly epitheliotropic viruses infecting skin or mucosal surfaces, and displaying a very high selectivity for the specific epithelium infected. To date, more than 85 HPV types have been characterized [2]. Genital HPV types are associated with a wide spectrum of morphological lesions [3]. HPV-6, -11, -42, -43 and -44 are associated with benign lesions (condylomas), whereas HPV-31, -33, -35, -51, -52 and -58 are detected more frequently in low squamous intraepithelial lesions (LSIL) and HPV-16 and -18 are predominant in high grade SIL (HSIL) and invasive carcinoma [4]. HPV-16 and HPV-18 DNA sequences are frequently integrated into the genome of cervical cancer cells, and this integrated HPV DNA is an indicator of poor prognosis [5,6]. The prevalence of HPV DNA in cervical lesions ranges from 25 to 90%. HPV-16 accounts for the highest proportion, followed by HPV-18, HPV-45 and HPV-31, but their incidence varies depending on the country [7].

The lack of standardized detection methods represents a major problem for monitoring HPV in developing countries, where the problem is most acute. In the absence of an international consensus concerning detection methods, HPV infections are difficult to diagnose. However, sensitive and specific methods, based on the detection of HPV DNA, are available. Among them, the polymerase chain reaction (PCR) technique is the method of choice for epidemiological studies [8]. However, PCR is a major technological advance not readily available in developing countries.

In a previous study, it was reported that HPV-16 is the most frequent type in Morocco, followed by HPV-18 [9]. Thus, we focused our interest on these two types. In Morocco, invasive cervical cancer is the primary cause of cancer deaths in women and represents a major public health problem. At the hospital-based cancer registry of the Institut

National d'Oncologie (INO) in Rabat, the number of cases of mostly advanced invasive carcinomas exceeds 500 per year [9]. The present study aimed to determine the type of HPV DNA present in inflammatory cervical lesions and invasive cervical carcinomas in patients from northern Morocco. Knowledge of the HPV types circulating in developing countries is very important for the future application of prophylactic vaccines.

PATIENTS AND METHODS

To determine the presence of HPV in cervical pathology in Rabat, we set up a PCR laboratory. Cervical biopsy specimens were obtained from 129 consecutive patients coming for consultation and treatment to the INO in Rabat, Morocco, between June 1999 and June 2000. Samples were obtained with informed consent according to INO policy [9]. The patients came from the northern area of Morocco, from Rabat to the Mediterranean coast, where half the population of the country is located. This is the geographical area covered by INO. There was no concentration of cases from any specific region in this area. There was no case selection because we wanted to determine what was present in the cancer patients in general. Two pathologists independently analysed all biopsies and sections were sent for molecular analysis to the Molecular Biology Unit of CNESTEN in Rabat. Lesions were classified according to FIGO (International Federation of Gynecoloy and Obstetrics) stages [10]. Therefore, they were enrolled as consecutive cases for which biopsy material was available. Only material from patients who had not yet been treated by radiation therapy was used.

DNA was extracted from paraffin-embedded sections (5–10 μm) with xylene and ethanol. The remaining cellular pellet was resuspended in digestion buffer (20 mM Tris–HCl pH 8.0, 20 mM ethylenediaminetetraacetic acid and 0.3 M, 0.5% sodium dodecyl sulphate and 0.1 mg/mL proteinase K) and incubated overnight at 37 °C. DNA isolation was performed by phenol–chloroform extraction and ethanol precipitation, and was resuspended in 50 μL of sterile distilled water [11].

The amplification reaction was performed in a final volume of 50 μL containing 50 pmol of each primer (MY09 and MY11), 200 μM each dNTP, 0.625 units *Taq* DNA polymerase (Amersham Biosciences, Little Chalfont, UK) and 3 μL of DNA sample in 1× *Taq* polymerase buffer. The mixture was first denatured at 94 °C for 7 min. Then, 35 cycles of PCR were performed by denaturation at 94 °C for 30 s, primer annealing at 52 °C for 10 min, and primer extension at 72 °C for 90 s. At the end of the last cycle, the mixture was incubated at 72 °C for 7 min. The PCR was performed in a thermocycler Amplitron II Thermolyne model (Barnstead Thermolyne Corporation, SYBRON INTERNATIONAL, Milwaukee, WI, USA). For every reaction, a negative control in which the DNA template was omitted from the amplification mixture, and a positive control were included. Aliquots of 10 μL of the PCR product were analysed by electrophoresis through a 1.2% agarose gel. The lengths of the amplified viral L1 fragment was the expected 450 base pairs (not shown), and to avoid problems associated with the use of HPV consensus primers [12], PCR products were transferred to a Hybond-N^+ membrane (Amersham Biosciences) and the membrane was hybridized with either a consensus HPV probe, or HPV-16- and HPV-18-specific probes [13]. The signal in the blots was detected using the DIG luminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions.

RESULTS

The histopathological analysis of the cases detected inflammatory lesions in 34 cases (26%), low grade cervical intraepithelial neoplasia (CIN) in eight cases (6%), and high grade CIN and invasive carcinomas in 87 cases (67%). An additional 20 cases with normal squamous epithelium were studied. It is important to draw attention to the disproportionately high number of advanced cases: 67 samples already corresponded to invasive carcinomas in stages II to IV, which are unusual in European or North American countries. In Morocco cervical lesions are usually diagnosed in very advanced stages of the disease.

HPV detection and typing were always performed in coded samples. The code was opened to identify the histopathological grade corresponding to each sample after the HPV result was available.

The results obtained for the 129 consecutive specimens are summarized in Table 1. HPV DNA was detected in 91 biopsy samples. Our results showed that HPV-16 was by far the most frequent type: it was found in 45 of the cases (49%) whereas HPV-18 was detected in only 20 cases

Table 1 Distribution of HPV types in consecutive cervical biopsies from northern Morocco

Lesion	No. of cases	HPV negative	HPV positive	HPV type		
				HPV-16	HPV-18	Other
Inflammatory lesions	34	9	25 (100)	10 (40)	6 (24)	9 (36)
Low grade CIN	8	4	4 (100)	4 (100)		
High grade CIN and invasive carcinomas (ISC and ADC)	87	25	62 (100)	31 (50)	14 (23)	19 (31)
Total	129	39	91 (100)	45 (49)	20 (22)	28 (31)

CIN, cervical intraepithelial neoplasia; ISC, invasive squamous cell carcinoma; ADC, adenocarcinoma.
Two ISC cases were HPV-16- and HPV-18-positive and are counted in both columns. In parentheses are the percentage values.

(22%). Two specimens had a double infection with HPV-16 and HPV-18, and 27 HPV-positive cases (30%) corresponded to other viral types that were not identified. The relative distribution of HPV-16 and HPV-18 in inflammatory lesions was similar to that in invasive cervical carcinomas (Table 1).

DISCUSSION

In Morocco, as in other developing countries, the lower social conditions, the average age at first intercourse, the high rate of parity and the lack of primary care in the health systems are important risk factors for the rate of cervical cancer [9]. The prevalence of HPV-16 in invasive cervical carcinomas from northern Morocco represented 49% of the HPV-positive specimens. This value is somewhat lower than the results found in other developing countries: Latin America (54.4%), Thailand (59.3%) and India (64%). In some European countries, the HPV-16 prevalence was higher, such as 78.3% in Poland and 76.5% in Germany [1].

In our study, HPV-18 was found in 22% of HPV-positive specimens, which is more than twice the level previously reported in Moroccan samples [9]. The relative proportion of HPV-16/HPV-18 in this study is similar to that for Africa and other countries such as Algeria, but is different from that in other Mediterranean countries in Europe, which have a higher proportion of HPV-16 cases [1]. We also found a similar virus-type distribution in inflammatory lesions and in cervical carcinomas (Table 1). These inflammatory lesions might be precursors of future cervical carcinomas, but this point will only be established after a follow-up of these patients for several years. The persistence of HPV DNA is an indicator of possible cervical cancer development in the future [14]. Thus the detection of high-risk HPV types in women is an important risk factor that allows identification of those women who should be monitored more closely [15], rather than concentrating limited medical resources on patients with lesions that will spontaneously regress. The age of determination may vary depending on the age structure of the population of a specific country.

The detection of a number of HPV-negative cases is a reality that must be fully recognized, instead of trying to attribute these cases to either a yet unknown HPV type or technical problems (both arguments are difficult to justify today). Cervical cancer shares with all other tumours the same biological characteristics regarding alterations in cell growth and division, apoptosis, cell life span, angiogenesis, tissue invasion and metastasis [16]; therefore cervical epithelial cells should also be able to undergo the same malignant processes as epithelial cells from other organs, which are independent of HPV presence. These properties have a genetic origin which is not HPV related, and that has been extensively studies in cervical carcinoma, sharing many characteristics, such as common loss of heterozygosity (LOH), with other tumors as expected [17]. However, HPV persistence is a major predisposing factor for tumor development in cervical cells containing HPV DNA [14,18], hence its detection in such a very high number of invasive cervical carcinomas. In many different studies, the number of negative cases ranges from 5 to 25%. Thus we can reasonably assume that approximately 10% of the cases are HPV negative, and therefore the role of persistent HPV will be to increase, by at least an order of magnitude, the likelihood of developing cervical carcinoma.

The magnitude of the public health problem represented by cervical carcinoma in developing countries might be even bigger in the future,

considering that the population of these countries is relatively young compared to that of developed countries. This means that if public health measures are not taken in order to identify the high-risk subpopulation, the burden of cervical carcinoma in these countries will increase considerably. HPV is currently the target of many vaccine development projects for prevention and therapy of cervical carcinoma [19]. The knowledge of HPV types circulating in developing countries is an essential component for the future application of prophylactic vaccines that aim to reduce the burden of cervical carcinoma [19,20]. In developing countries the control of HPV by vaccination is likely to be a more effective and practical approach than the implementation of regular and periodic cytological screening. Currently prophylactic vaccines are based on HPV-16 [20,21]. The virus-type distribution varies among different regions of the world, which means that there will always be a proportion of potential cases that will not be prevented. It is known that vaccination using virus-like particles against L1 from HPV-16 induces a significant immune response, which does not reduce the likelihood of acquiring another virus type [22]. Furthermore, the persistence of virus types not targeted for vaccination might represent a new problem with time [23], since they might occupy the biological niche left empty by the vaccine, and a previously low-frequency malignant type might eventually become dominant and widespread.

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