

Detection of human papilloma virus in women attending the IRCCS , Ospedale oncologico Bari, Southern Italy: preliminary data

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SUMMARY

We investigated the diffusion of HPV genotypes using molecular methods. HPV DNA was detected in 30.4% of women examined. The genotype HPV 16 was the most common followed by HPV 31, HPV 51 and HPV 58. Mixed infections were observed in 30.4% of HPV positive women. The 66.7% of the lower age group (<35 years) was HPV positive. HPV infection was associated with the presence of morphological abnormalities in 13.7% of the women examined. The presence of HPV DNA in women younger than 35 years is an indication for the implementation of sexually transmitted disease education in our area to prevent potentially dangerous infections.

KEY WORDS: HPV, HPV genotypes, morphological abnormalities

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INTRODUCTION

Human papillomaviruses (HPVs) are a heterogeneous group of viruses that infect skin and mucosal epithelial tissues and play an important role in cervical carcinogenesis (Kjaer *et al.*, 1996). In humans, more than 100 genotypes have been identified by molecular studies (Molijn *et al.*, 2005; de Villers *et al.*, 2004). HPV can be divided into low-risk HPV (LR-HPV) or high-risk HPV types (HR-HPV). LR-HPV have mostly been found in benign lesions and low grade squamous intraepithelial lesions (LSILs), whereas some HPV genotypes (mostly HPV-16 and 18, but also HPV-31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 66, 68, 70 and others) have been classified as HR-HPV types on the basis of their great prevalence (80-90%) in high grade squamous intraepithelial

lesions (HSILs) and in cervical cancer (Munoz 2003).

The purpose of this preliminary study was to use a biomolecular method to investigate: 1. The possible diffusion of HPV and the genotypes most frequently present; 2. the type of cervical epithelium lesions associated with HPV.

This study was conducted over a two-year period (2002-2003) on 102 women (mean age of 46.3 years, range 22-78 years) referred to the IRCCS Ospedale Oncologico, Bari, Southern Italy for the presence of either cervical lesions observed in cytological and/or histological samples, or a history of genital warts, or for cervical screening. Various kinds of specimens were considered. Cervical specimens were collected by brushing from all 102 women; the samples were stored at +4°C until testing. Paraffin embedded bioptic specimens or samples collected at surgery were also evaluated in 67 of these women.

Cases were classified according to the Bethesda system for the reporting of cervical/vaginal cytological diagnoses into: normal, low grade squamous intraepithelial lesions (LSIL) for mild dysplasia, high grade squamous intraepithelial

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lesions (HSIL) for moderate and severe dysplasia and invasive squamous carcinoma (Solomon *et al.*, 2002). Histological diagnosis was reported as normal, cervical intraepithelial neoplasia (CIN) was classified in three grades: CIN 1, CIN 2 and CIN 3 for mild, moderate and severe dysplasia respectively and finally invasive squamous cell carcinoma (Richart, 1980). Patients were divided by age into two classes: 35 years old and over, and younger than 34; 84 women were in the older group and 18 in the younger.

DNA was extracted from each sample by the "Extracell" kit (BIOLINE, Italy) according to the manufacturer's protocol. HPV was detected using the "HPV Screening" kit (BIOLINE, Italy) in which consensus primers were used. The MY09-MY11 consensus primers contain a mixture of 24 primers able to amplify a 450-bp fragment within the conserved L1 region common to numerous HPV types (Gravitt *et al.*, 1998). Rates of PCR positive samples were further analyzed using primer pairs specific for the high and low risk E6/E7 HPV regions by the "HPV Typing" kit (BIOLINE, Italy). The cycling protocol for E6/E7 HPV region was performed according to the manufacturer's instructions and PCR products were analysed by electrophoresis in 4% agarose gel. Amplicons from HPV-positive samples were subsequently genotyped by a reverse hybridization assay, using the "INNO-LiPA HPV Genotyping" kit (Immunogenetics N.V., Belgium) which permits the specific detection of 25 HPV genotypes (Kjaer *et al.*, 1996). This system uses a general primer set, designated SPF₁₀ that amplifies a 65-bp segment of the L1 region of the HPV genome (Kleter *et al.*, 1998). Statistical analysis was performed using the χ^2 Test. The values were considered significantly different when the P value was <0.05.

HPV DNA was detected in 31 of 102 women (30.4%). When these 31 positive samples were analyzed for the high and low risk E6/E7 HPV region, 7 (22.6%) were LR-HPV and 24 (77.4%) were HR-HPV.

In 4 patients with evidence of HR-HPV and in 4 patients of LR-HPV infection, it was impossible to identify the HPV genotype with LiPA. In the other 23 samples, the following types were identified: 6, 11, 40, 53 of LR-HPV and 16, 18, 31, 33, 35, 39, 45, 51, 56, 58, 66, 68 of HR-HPV. The genotype HPV16 was the most

common (39.1%) followed by HPV 31, HPV 51 and HPV 58.

Sixteen (69.6%) of the 23 HPV DNA-positive samples genotyped by LiPA contained a single HPV genotype while mixed infections were observed in 7 cases (30.4%). In 4 patients an association of HR-HPV type was observed with the following association of HPV type: 16-51, 39-66, 31-45-68, 51-58-66. In the remaining three patients a mixed infection of both LR- and HR-HPV (31-58-40 in two cases and 8-51-11 in one case) was detected.

Twelve (66.7%) of the 18 women in the lower age group, were HPV positive whereas 19 (22.6%) of the 84 women of the upper age group resulted HPV positive (χ^2 : p=0.0006). HR-HPV types were detected in 83% of the lower age group.

HPV infection was associated with the presence of morphological abnormalities in 14 (13.7%) out of 102 patients (Table 1). Among these 8 had a histological diagnosis of LSIL, 2 of HSIL and 4 of invasive carcinoma. In LSIL lesions, HR-HPV was identified in 6 patients, and LR-HPV was present in the other two. In HSIL lesions and in invasive carcinoma only HR-HPV was present. HPV 16 and HPV 33 were detected in three and one patients with carcinoma, respectively (Figure 1).

Both lesions and HPV DNA were absent in 67 (65.7%) of patients.

In 17 (16,6%) HPV positive women, no abnormalities were found. Twelve of these were infected with HR-HPV and five with LR-HPV. Moreover the presence of abnormal cells was associated with the absence of HPV DNA in 4 women. Two patients had only a cytological diagnosis of LSIL, two had a histological diagnosis of squamous cell carcinoma and adenocarcinoma, respectively.

TABLE 1 - The significant agreement between cytologic abnormalities and presence of HPV DNA

Lesions	HPV	
	presence	absence
presence	14	4
absence	17	67

p ≤ 0.05

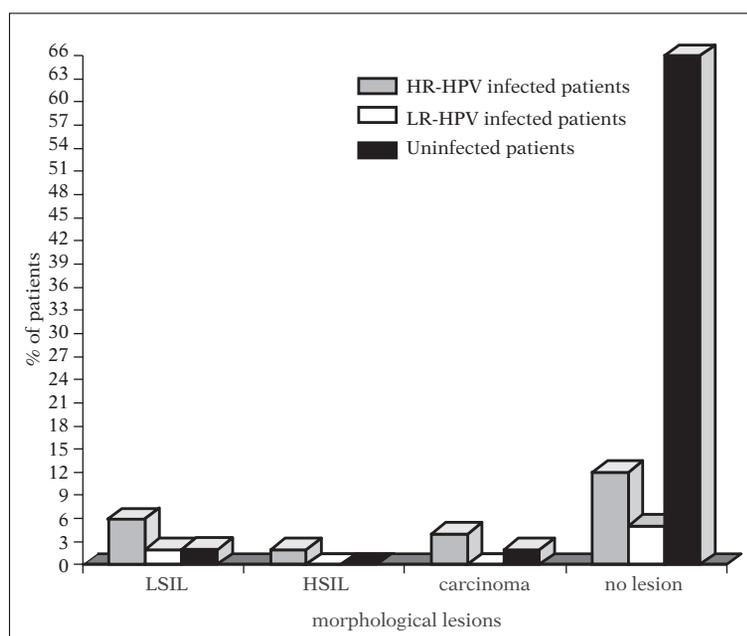


FIGURE 1 - Relationship between HPV status and cytological findings.

All these data show that HPV infection was present in 77.7% of women with morphological abnormalities.

In developing countries, cervical cancer is common and may constitute up to 25% of all female cancer. Over 30 genotypes of genital HPV are reported to be oncogenic. Of these, four are frequently found within the neoplastic cells, with type 16 accounting for about half of these cases in the United States and Europe. Types 18, 31 and 45 account for an additional 25-30% of cases (Harro *et al.*, 2001; Anhang *et al.*, 2004).

In our experience, sixteen different HPV genotypes have been detected. HPV-16 was the most prevalent type in agreement with that reported in the literature (Spano *et al.*, 2005; Kay *et al.*, 2005)

In 8 patients we were unable to identify the genotype by LiPA. If this was due to technical errors or to the low sensitivity of INNO-LiPA in detecting some HPV genotype such as 42 and 59 (van Doorn *et al.*, 2002) or to the presence of HPV types not detectable by LiPA remains to be established (Jung *et al.*, 2004).

HPV-DNA was not found in 4 women with cell abnormalities. The analytical sensitivity of the PCR using the MYO9 plus MY11 primers is between 10 and 200 HPV copies depending on the HPV type (Burd, 2003). False-negative PCR results are reported to be 7% in cervical cancer

(Volpers *et al.*, 1991) and to be in the range 1.1-7.5% using the Hybrid capture assay system (Burd, 2003). Our negative results may be due to several factors such as the low level of infection, sampling error, the presence of interfering substances. Of these four HPV negative women two had LSIL, one an adenocarcinoma, and one only a squamous carcinoma.

It has been reported that HR-HPV DNA is detected in 83% of LSIL women; our percentage of negative results is about 20% and is in agreement with this value notwithstanding the low number of women studied in this preliminary report. The negative result in the woman with a carcinoma may be because of the absence of HPV DNA in the carcinoma cells, or a false-negative PCR result due to integration of HPV DNA in the cervical carcinoma which may have disrupted PCR primer target sequences or resulted in a loss of the L1 ORF (L1 Open Reading Frame) targeted by the consensus primers used. These factors may also explain the absence of HPV in the woman with adenocarcinoma even if the association of HPV and this pathology is not well established (Chew *et al.*, 2005).

Although a high percentage of infected women clear the infection by immunological mechanisms, a small percentage of high-risk HPV infections progress to cervical cancer. The risk of

acquiring genital HPV infection is influenced not only by sexual activity, but also increases with age (Roden *et al.*, 1997; Adam *et al.*, 2000). In our study the prevalence of HPV DNA was highest (66.7%) in women under age of 35 and significantly lower (22.6%) in women of 35 or older. Young women have a probability of acquiring a HPV infection about seven times higher than that of older women (Evander *et al.*, 1995).

HPV infections most commonly occur in sexually active young women, 18 to 30 years of age, whereas cervical cancer is more often found in women over 35, indicating that HPV infection at younger ages can result in a slow progression to cancer (Jung *et al.*, 2004; Burd 2003; Kahn *et al.* 2004).

In the present study, we tested a total of 102 samples for the presence of HPV DNA by PCR in combination with LiPA assay. Moreover, the use of the two methods also allowed us to detect the HPV genotype associated with the presence of low grade of lesions or no lesions and to determine the presence of multiple or single genotypes in the same patient. The awareness of the genotypes involved in infection may be useful to prevent HPV infections through the development of a vaccine, the most promising way of controlling cervical neoplasia at this time (Zhang *et al.*, 2000). In addition the detection of HPV coinfection is important as various studies have demonstrated that infection with multiple HR-HPV type tends to increase the severity of cervical cancer (Quint *et al.*, 2001). The presence of HPV DNA in the 66.7% of women younger than 35 is an indication for the implementation of sexually transmitted disease education in our area. The detection of HPV DNA in women, also in the absence of cytological lesions, suggests that HPV-positive women must be monitored over time to make an earlier diagnosis of pre-cancerous lesions and confirm the importance of HPV detection in screening programs.

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