

Prevalence and type distribution of human papillomavirus infection among women with different degrees of cervical cytological abnormalities in Sicily (Italy)

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Summary

Human papillomaviruses (HPVs) are etiological agents of cervical cancer. In the absence of Pap smear alterations, high-risk HPV DNA can be detected in cervical samples. The prevalence of papillomavirus

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Contributions: CF designed the experiments, participated in performing the molecular tests, performed the interpretation of data, and drafted the manuscript; CMC conceived the study, performed the molecular tests and the interpretation of data, and drafted the manuscript; RR, CIP participated in interpretation of data and helped to draft the manuscript; GS participated in the design of the study, supervised and coordinated it, and participated in drafting the manuscript. All authors read and approved the final manuscript.

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infection and their genotype distribution varies greatly across populations. The aims of this study were: i) to assess the prevalences of HPV genotypes in people living in Eastern Sicily (Italy) and the frequency of HPV multiple infections; ii) to evaluate the association between HPV genotypes and cervical lesions in order to improve the epidemiological knowledge useful for monitoring or treating infected women. Nested PCR and reverse dot/blot hybridization were used for the detection and typing of HPV DNA in 315 women who had had an abnormal PAP-smear. HPV DNA test was positive in 70.5% cases; the prevalence was 50% in atypical squamous cells of undetermined significance (ASCUS), 80.8% in low grade-, and 76.2% in high grade-squamous intraepithelial lesion (H-SIL). The genotype distribution showed a predominance of HPV-16 (56.7%) followed by HPV-18 (12.2%), HPV-31 (9.5%) and HPV-6 (9.5%). Multiple infections were detected in 35.1% of the infected patients. High frequency of positive results for HPV was confirmed and, even in case of ASCUS, patients should be taken into account for genotyping. Our data indicate that multiple infections are consistent in women with low-grade lesions while they are less frequent in women with H-SIL. This could reinforce the theory of the multi-stage cancer model, by which one HPV type becomes predominant along with the progression of cervical lesion severity.

Introduction

Genital human papillomavirus (HPV) infection is the most common sexually transmitted viral infection worldwide (3). It is associated with various clinical conditions, ranging from asymptomatic infections to benign or malignant diseases of the genital mucosa (7).

More than 100 human papillomavirus (HPV) genotypes are known, and more than 40 of these can infect the anogenital area either in men or in women. On the basis of the phylogenetic and epidemiological characteristics, they are grouped as low-risk HPV (LR-HPV), commonly associated with low-grade cervical lesions, and high-risk HPV (HR-HPV), associated with high-grade lesions and/or cancer (5,28). Genotype distribution may vary globally and within geographical areas. In a Planned Parenthood population in the United States with a mean age of 25 years, the prevalence of high-risk HPV infection was 27.4% (17). Similar prevalence estimates were found among female university students in the U.S. and Canada (16). In Europe, the most prevalent genotype is HPV 16, followed by HPV 31 and HPV 66 in Eastern and Southern Europe, respectively (12). A recent study showed that in Scotland the prevalence of PCR-detected HPV DNA in women (mean age 36.6 years) attending routine cervical screening was approximately 20.5% for all HPVs and 15.7% for HR-HPVs (10). In a study run in

Barcelona, HPV DNA was detected in 68% of a cohort of women followed due to cervical dysplasia (24).

At present, little is known about the prevalence and distribution of HPV genotypes in Italy. Only a few studies, mainly based on Northern Italy populations, have investigated the prevalence of cervical HPV infection in healthy women (6,11,21) and in women with cytological abnormalities (15,19,27).

The aims of this study were: i) to assess the prevalences of HPV genotypes and the frequency of multiple infections in people living in Eastern Sicily; ii) to evaluate the association between HPV genotypes and cervical lesions in order to improve the epidemiological knowledge useful for monitoring or treating infected women.

Materials and Methods

Study population

This is a prospective study that included a cohort of 315 women, who were referred to the Clinical Virology Unit of the Central Laboratory, University Hospital *Policlinico-Vittorio Emanuele, G. Rodolico* of Catania, Italy, from February 2009 to February 2010.

The mean age of the patients was 34.5 years (range, 18-65 years).

All women included in this study had had a PAP smear classified as abnormal according to the Bethesda System (TBS, 2001) that reports cervical cytological diagnoses: atypical squamous cells of undetermined significance (ASCUS), low grade squamous intraepithelial lesion (L-SIL), and high grade squamous intraepithelial lesion (H-SIL). Thus, women were subdivided into three groups: 96 (30.5%) were classified as ASCUS; 156 (49.5%) as L-SIL; and 63 (20.0%) as H-SIL.

Sample collection

Endocervical cytobrush or cervical swab was collected and suspended in 20 mL of ThinPrep® Pap Test PreservCyt® Solution (Hologic Inc, MA, USA). The solution was placed into two tubes and pelleted by centrifugation at 1000 rpm for 20 min. The supernatant was removed and discarded and the pellet was resuspended in 2 ml aliquots in 2 tubes and stored at -20°C until use.

Human papillomavirus DNA extraction, amplification, and genotyping

The automated DNA extraction was performed with 1 ml of sample on the NucliSens easyMAG system (bioMérieux SA, Marcy l'Etoile, France) following the manufacturer's HPV 1.1 protocol, with a 55 µL final elution volume.

Amplification of HPV DNA was accomplished by nested PCR using HPV-HS Bio kit (AB Analitica s.r.l., Padova, Italy) according to the manufacturer's recommendations.

For the first-amplification step, performed with 10 µL of eluate, a combination of degenerate primers was used to amplify a 449-458 bp sequence within the L1 ORF of the HPV genome. The second amplification was performed from 1 µL of the first amplification product, using biotinylated primers to amplify a 139-145 bp sequence. To verify the efficiency of the DNA extraction, 10 µL of eluate were used to amplify a 202 bp fragment of the Thiosulfate SulfurTransferase (TST) gene using specific primers. Negative (water) and positive controls (plasmid clones containing HPV 54) were included in each PCR run, to check for accuracy or possible contamination. The product of the second amplification and of the TST were detected and identified by electrophoretic separation into a 2% agarose gel with 2,5 mg/ml ethidium bromide in TAE buffer. A specimen was considered positive if a 202 bp band (TST) and of 139-145 bp band (HPV target) were detected by an UV transilluminator. The positive specimens were used for the hybridization step; those negative for TST were considered inadequate and extracted again from the second tube.

Finally, HPV typing was performed with a reverse line blot hybridization assay with specific probes for the most frequent HPV-types (Ampliquality HPV-type assay, AB Analitica s.r.l., Padova, Italy). HPV-type allows the identification of 11 LR-HPV (HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81) and 18 HR-HPV or probably HR-HPV (HPV-16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -73, -82).

Samples that were positive by gel electrophoresis but negative in reverse line blot for any of the identifiable types were considered as HPV undetermined.

Results

HPV DNA was detected in two hundred and twenty-two patients (70.5%) distributed differently in the cytological categories: the HPV prevalence estimates were 50% (48/96) in women with ASCUS, 80.8% (126/156) in women with L-SIL, and 76.2% (48/63) in women with H-SIL.

Table 1 shows the distribution of the results of the HPV detection related to cytological abnormalities.

Multiple HPV infection was found in 35.1% of the HPV DNA positive subjects; in particular, 31.2% of ASCUS, 40.5% of L-SIL, and 25% of the H-SIL groups patients were infected by two or more HPV genotypes.

Among the 222 HPV DNA positive patients, the prevalence of HR-HPV genotypes increased with the severity of cytology: 36 (75.0%) ASCUS and 104 (82.6%) L-SIL women were positive for HR-HPV, with multiple infections in 15 and 51 cases, respectively. In all H-SIL women were detected HR-HPV, with multiple infections in 12 cases: 9 in combination with at least another HR-HPV and 3 with LR-HPV.

HPV 16 was the most prevalent genotype in all the categories considered: 31.3% (15/48) of ASCUS, 52.4% (66/126) of L-SIL, and 93.5% (43/48) of H-SIL.

Table 1. Distribution of human papillomavirus DNA detection related to cytological abnormalities.

	ASCUS, n. (%)	L-SIL, n. (%)	H-SIL, n. (%)
Negative	48 (50.0)	30 (19.2)	15 (23.8)
HR S.I.	21 (43.8)	53 (42.1)	36 (75.0)
LR S.I.	7 (14.6)	13 (10.3)	0
UD	5 (10.4)	9 (7.1)	0
M.I.	15 (31.2)	51 (40.5)	12 (25.0)
Total	96 (30.5)	156 (49.5)	63 (20.0)

ASCUS, atypical squamous cells of undetermined significance; L-SIL, low grade squamous intraepithelial lesion; H-SIL, high grade squamous intraepithelial lesion; S.I, single infection; UD, undetermined; M.I, multiple infections.

HPV 18 was detected in 27 patients (12.2%), in association with other genotypes in 21 cases.

After HPV 16 and 18, the more prevalent genotypes were HPV 31 (9.5%) and HPV 6 (9.5%) in all the categories considered (Table 2).

Discussion and Conclusions

In this study, we observed the distribution of different HPV genotypes in a cohort of 315 women from Eastern Sicily.

HPV was detected with high frequency not only in women with H-SIL, but also in women with low-grade lesions (ASCUS and L-SIL).

HPV 16 was the genotype most frequently detected in all studies reported in literature in either single or multiple infections with other genotypes.

Owing to its association with more than 50.0% of all cervical cancers (22), the prevalence of HPV 16 in the female populations gains particular interest. Compared to other HPV types, the frequency of detection of HPV 16 can differ among countries (26). In the present study HPV 16 was the more common HR-HPV (56.7%) revealed with a prevalence increasing with the severity of abnormal cervical cytology (31.3% in ASCUS, 52.4% in L-SIL, and 93.5% in H-SIL).

HPV 16 is a persistent genotype, it is highly carcinogenic, and it can be integrated into the host genome, becoming one of the major contributing factors to genital malignant transformation (14,18).

From our results it appears that HPV 16 (56.7%), 18 (12.2%), 6 (9.5%), and 31(9.5%) were the most prevalent genotypes followed by HPV 52 (6.8%), and 59 (6.8%).

HPV 45, considered as the third most common genotype in invasive cervical cancer, was never found in the examined positive women.

HR-HPV positivity rates of 75.0%, 82.6% and 100%, found in women with ASCUS, L-SIL and H-SIL respectively, in the present study are largely comparable with those reported in the literature (8,25,1).

Moreover, the high frequency of detection of HR-HPV in ASCUS

group, suggests to screen also the ASCUS cases for HPV DNA. Finally, the finding of HPV 16 positivity in 93.7% of the H-SIL cases, in either single or multiple infection, suggests that the probability of progression from ASCUS to H-SIL or invasive cancer in women infected with HPV 16 may be quite high and so far underestimated. As reported by Anderson (2), the sensitivity of the HPV DNA test is higher than a repeated Pap smear; thus HPV testing had a very high negative predictive value (99.5%) in women with an ASCUS Pap smear. This indicates that if an HPV test is negative, cervical disease due to HPV is highly unlikely. These observations support HPV DNA testing as viable option in the management of women with ASCUS cytology.

The role and the impact of multiple HPV infections on the risk of cervical dysplasia is controversial (9,13,20). Becker *et al.* (4) suggested that multiple HPV infections carry an increased risk of dysplasia only in patients with HPV 16. We observed that multiple infections are common and the proportion of multiple infections decreases in H-SIL (25.0%) if compared with ASCUS (31.2%) and L-SIL (40.5%), as reported by Agarossi *et al.* (1).

This observation reinforces the theory of the multi-stage cancer model, by which one HPV type tends to predominate with the rise of cervical lesion severity (23).

Further studies are on-going to define the role of HPV DNA testing in the management of cervical disease due to HPV infection.

Although the Pap test is an important diagnostic tool in cervical-vaginal screening for its high sensibility, it has the disadvantage of not being able to reveal the infection with HR-HPV in the absence of cytological abnormalities.

Current molecular technologies, including HPV DNA detection and the E6/E7 mRNA test, which provides important information about the potential progression of HPV infection, represent a more accurate, sensitive and specific, diagnostic approach as well as a more valid method for post-treatment follow-up of women with significant cytological abnormalities.

In this way the pre-cancerous lesions can be identified and treated precociously in order to lessen the physical and psychological impact on women.

Table 2. Prevalences of the different genotypes in the cytological categories.

Genotypes	ASCUS (50.0%)	L-SIL (80.8%)	H-SIL (76.2%)	TOT. (70.5%)
6	11.1	14.3	-	9.5
40-42-69	11.1	7.1	-	5.4
43-61-70	11.1	9.5	-	6.8
16	31.3	52.4	93.5	56.7
18	18.7	9.5	12.5	12.2
31	18.7	9.5	-	9.5
33	-	4.8	6.2%	4
35	-	-	6.3%	1.3
39	11.1	2.9	6.2%	4
51	-	2.9	-	1.3
52	11.1	9.5	-	6.8
53	11.1	7.1	-	5.4
56	-	4.8	-	5.4
58	11.1	7.1	-	5.4
59	11.2	9.5	-	6.8
66	11.1	4.8	-	4
73	-	7.1	-	4
U.D.	12.5	7.1	-	6.8

ASCUS, atypical squamous cells of undetermined significance; L-SIL, low grade squamous intraepithelial lesion; H-SIL, high grade squamous intraepithelial lesion; UD, undetermined.

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