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Prevalence of Alpha-Papillomavirus Genotypes in Cervical Squamous Intraepithelial Lesions and Invasive Cervical Carcinoma in the Italian Population

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The aim of the present investigation was to define the spectrum of mucosotropic human papillomaviruses among 414 Italian women with normal cervices (n = 183), low- and high-grade cervical squamous intraepithelial lesions (n = 101 and 65, respectively), and invasive squamous cervical carcinomas (n = 65). Human papillomaviruses were detected by broad spectrum consensus-primer-pairs MY09/MY11 and GP5+/GP6+-based polymerase chain reaction using three amplification methods and were characterized by nucleotide sequence analysis. The prevalence rates of HPV infections was 19.7%, 63.4%, 80%, and 81.5% in patients with normal cervices, low-grade, and high-grade squamous intraepithelial lesions, and cervical carcinomas, respectively. Among the 205 HPV-positive patients, a total of 31 mucosal HPV genotypes were identified of which 16 types, epidemiological classified as high-risk viruses (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 66, 68, 73, and 82), have been found in 16.9%, 50.1%, 69.2%, and 78.5% of normal cervix, low-, and high-grade cervical squamous intraepithelial lesions, and cervical carcinoma groups, respectively. As expected, the HPV16 was the most represented viral type in all groups examined with frequency rates ranging from 8.7% in normal subjects to 58.5% in invasive carcinoma patients. Ten epidemiologically defined low-risk HPV types (HPV6, 11, 42, 54, 61, 70, 71, 72, 81, 83) were detected in 2.7%, 7.9%, and 6.1% of normal cervix, low-, and high-grade cervical squamous intraepithelial lesions, respectively, and in none of invasive carcinomas. Furthermore, five unknown risk viruses were detected in 3% of low-grade cervical squamous intraepithelial lesions (HPV30, 32, 67), in 3.1% of high-grade cervical squamous intraepithelial lesions (HPV62, 90), and in 1.5% of cervical carcinomas (HPV62). Larger epidemiological screening studies, with PCR amplification and followed by either hybridization-based procedures against sequence targets of all known HPV types or sequence analysis studies, are

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needed in order to assess the epidemiological risk of less represented HPV types, to identify unknown viruses, and to monitor the future eventual spread of unusual viral types related to vaccination programs and/or population mobility. ***J. Med. Virol. 78:1663–1672, 2006.***

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INTRODUCTION

Human papillomaviruses are a large and heterogeneous group of viruses comprising almost 100 fully characterized genotypes and more than 100 putative new HPV types defined on the sequence analysis of subgenomic amplicons [zur Hausen, 2000; de Villiers et al., 2004]. Fifty different HPV types, grouped in the genus alpha papillomavirus, are known to infect the anogenital mucosa. Molecular epidemiologic evidence clearly indicates that 15 alpha HPV types clustered by the L1-based phylogenetic taxonomy in the species number 5 (HPV51), number 6 (HPV56, 66), number 7 (HPV18, 39, 45, 59, 68), number 9 (HPV16, 31, 33, 35, 52, 58), and number 11 (HPV73), are related causally with high-grade squamous intraepithelial lesions and invasive cervical carcinomas. On the other hand, 11 HPV types, grouped in species number 1 (HPV42), number 3 (HPV61, 72, 81), number 10 (HPV6, 11, 44), number 8 (HPV40, 43, 91), and number 13 (HPV54), are found mainly in genital warts and low-grade squamous intraepithelial lesions [de Villiers et al., 2004]. Therefore, according to their malignant potential, these groups of viruses have been classified as high- or low-risk types [van Ranst et al., 1992; Munoz et al., 2003]. Three uncommon HPV types, belonging to high-risk species number 5 (HPV26) and number 6 (HPV53 and 66), have been classified as probable high-risk types on the basis of their detection in few malignant lesions and no controls [Munoz et al., 2003]. The epidemiology and risk estimates and the biological properties for latter viral types along and for many other less represented HPV types remain still unknown. As a consequence, larger epidemiological studies with appropriate detection methods are needed to assess their role in genital tumors. Most studies on the natural history of HPVs and their role in the development of genital cancer have employed the L1 consensus primer PCR systems, particularly the MY09/MY11 and GP5+/6+ primer pairs [Resnick et al., 1990; de Roda Husman et al., 1995], which amplify a broad spectrum of HPV genotypes. Their variable level of sensitivity among different genotypes, however, underestimate of the true prevalence of HPV in clinical samples [Qu et al., 1997; Husnjak et al., 2000; Chan et al., 2006].

The recent success of monovalent (HPV16) and polyvalent (HPV6, 11, 16, and 18) virus-like particles-based vaccines in preventing persistent HPV infection and associated cervical lesions is a promising tool for the prevention of cervical cancer [Koutsky et al., 2002; Harper et al., 2004, 2006; Villa et al., 2005]. The vast heterogeneity of HPV infections, however, mandates the development of vaccines targeting a wider range of HPV types, considering that an adequate humoral response with neutralizing antibodies is essential as a primary effect of a prophylactic vaccine and that there is little cross-reactivity between high-risk HPV types belonging to the same phylogenetic group and no reactivity between unrelated viral types [Kirnbauer et al., 1994; Sasagawa et al., 1998; Ho et al., 2004].

Several studies described the heterogeneous distribution of HPV genotypes in different geographical regions [reviewed in and Munoz, 2000; Bosch and de Sanjose, 2003]. Thus large population-based epidemiological screening is necessary in order to establish which types are present in the various populations and which are associated with disease progression. With this in view, the International Agency for Research on Cancer (IARC) has coordinated an international survey on HPV type distribution in cytological normal women selected at random from the general population of 11 countries [Clifford et al., 2005a,b], and has undertaken meta-analysis studies on selected citations extracted from *Medline* and *ISI current contents* in order to evaluate the HPV type distribution in high-grade cervical lesions and invasive cervical carcinomas in countries representative of five continents [Clifford et al., 2003a,b]. All studies confirmed that while HPV16 with different frequency rates is the most commonly identified genotype in squamous intraepithelial lesions as well as in cervical carcinomas from all over the world, HPV genotypes other than HPV16 showed different prevalence rates varying by region. Among Italian women, a random sample of more than 1,000 subjects from Turin was studied with a detection method able to adequately identify a wide range of HPV genotypes [Ronco et al., 2005]. Few other such studies, however, have assessed only the prevalence of most common high-risk viral types (HPV16, 18, 31, 33, and 35) but not all characterized the spectrum of >40 mucosotropic HPV types in squamous intraepithelial lesions and cervical carcinoma tissues.

To complement these studies, a prevalent case-control study was undertaken of HPV prevalence and type-specific distribution among women, recruited in Northern (Milan) and Southern Italy (Naples), with normal cervix, low-, and high-grade squamous intraepithelial lesions as well as with invasive cervical carcinoma. The HPV DNA has been amplified by PCR based on broad spectrum MY09/MY11 and GP5+/GP6+ primer pairs using three different protocols in order to obtain the best amplification sensitivity for the different HPV genotypes, and genotyped by direct sequence analysis in order to identify uncommon HPV types that may not be recognized by hybridization-based typing methods used commonly.

SUBJECTS AND METHODS

Study Population and Samples

The present study includes 458 women with pre-malignant (low-grade and high-grade cervical squamous intraepithelial lesions) and malignant (invasive cervical carcinoma) cervical lesions (n = 251), as well as controls (n = 207) (normal cervical mucosal epithelia exfoliates of clinically healthy subjects). All women attended the Gynecology Divisions of the respective National Cancer Institutes in Milan (n = 109) and in Naples (n = 341), from January 1998 through December 2004. These Institutions are the regional reference centers for cancer patients and the local center for cancer screening programs. Case patients were selected from women with abnormal Pap smear following selection criteria: age >18 years; number of total partners <4; Italian ancestry >2 generations for the enrolled subjects and for all their sexual partners; absence of a previous history of sexual diseases or gynecological treatments; negative for human immunodeficiency virus (HIV) infection, and absence of infection risk factors, including intravenous drug usage.

Cervical cell scrapings were collected with a cytobrush from the ecto- and endocervix of the uterus of each woman. After obtaining smeared cell slides for Pap test, the remaining cell samples on the cytobrush were suspended in 1 ml of lysis buffer (10 mM Tris-HCl pH 7.6, 5 mM EDTA, 150 mM NaCl, 1% SDS) and stored at −20° until testing. All colposcopically visible lesions were biopsied and the specimens were fixed routinely in formalin, embedded in paraffin, stained with hematoxylin–eosin for histopathological diagnosis and stored at room temperature until analysis. All abnormal smears and/or histological slides were reviewed independently and the final diagnoses were determined by agreement of two pathologists using the Bethesda system.

All cases were graded as low-grade squamous intraepithelial lesions [encompassing mild dysplasia and cervical intraepithelial neoplasia grade 1]; high-grade squamous intraepithelial lesions [cervical intraepithelial neoplasia grade 2 and 3, and carcinoma in situ], and invasive squamous cell carcinoma according to the highest grade present within a lesion. Fifty invasive squamous cell cervical carcinoma biopsies, obtained from 50 patients who met the selection criteria and who had been referred to the above institutions in the same 4-year period, have been included in the study.

Selection-criteria-matched controls were chosen from women with normal cytology participating in the cancer screening programs. Controls (n = 207) were matched to cases on age (±3 years) and date of recruitment (±5 months).

All women gave voluntarily their consent to participate in this study. The study protocol was approved by the local ethical review board of the Institutions involved.

Genomic DNA was extracted from cervical-scraping cell lysates as well as from thin sections of fixed, embedded tissue according to published procedures [Tornesello et al., 1997; Fredricks and Relman, 1999; Buonaguro et al., 2000]. In particular, tissue biopsies needed a pre-DNA extraction treatment, characterized by processing of two 5 μm sections for each biopsy twice with 1 ml of xylenes and twice with 500 μl of 100% ethanol. DNA extraction was carried out by similar procedures for both types of samples (cervical scrapings and tissue sections); briefly samples were digested with Proteinase K (150 μg per ml at 60°C for 30 min) in 100 μl of lysis buffer (10 mM Tris-HCl pH 7.6, 5 mM EDTA, 150 mM NaCl, 1% SDS), followed by DNA purification by phenol and phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation in 0.3 M sodium acetate (pH 4.6). Tissue sections as well as cervical scrapes were analyzed for 50 cervical intraepithelial neoplasia grade 1 to 3 patients; HPV detection agreement between the two specimens was obtained in 49 cases (98%).

DNA quality test, carried out by amplification with specific oligoprimers targeting a fragment of the exon 7 within the *TP53* gene (Buonaguro et al., 1996), and DNA quantity analysis, evaluated by spectrophotometric measurements, provided 231 cases and 183 controls suitable for the viral DNA detection.

PCR Amplifications

HPV detection was carried out by (1) single round PCR methodology, using in parallel the MY09/MY11 and GP5+/GP6+ oligonucleotides to amplify the L1 conserved region as described by Resnick et al. [1990] and de Roda Husman et al. [1995], respectively; and (2) nested PCR methodology using as outer pair the MY09/MY11 oligoprimers and as inner pair the GP5+/GP6+ oligoprimer. Both outer and inner PCR amplification reactions were undertaken in 50-μl reaction mixture containing 20 pmoles of each primer, 50 mM KCl, 3.75 mM $MgCl_2$, 100 mM Tris-HCl pH 8.3, 0.1% Triton X-100, 50 mM of each dNTP, and 1.8 units of thermostable AmpliTaq DNA polymerase (Applera, Courtaboeuf, France). In the first-step PCR, 5 μl of DNA (10 to 500 ng extracted from cell scrapes to paraffin embedded tissues or fresh biopsies, respectively) was used as target DNA (outer reaction); in the nested PCR, 5 μl of the first-step PCR was used as input of amplified DNA (inner reaction). MY09/MY11 outer amplification reactions were performed in a Perkin-Elmer GeneAmp PCR System 9600 thermal cycler with the following steps: an initial 1-min denaturation at 94°C, followed by 32 cycles of 55°C for 1 min, 72°C for 2 min, 94°C for 30 sec, and a final annealing at 55°C for 1 min with 5-min elongation at 72°C. GP5+/GP6+ inner amplification reactions were performed with an initial 1-min denaturation at 94°C followed by 40 cycles of 40°C for 1 min, 72°C for 1 min, 94°C for 1 min, and a final annealing of 40°C for 1 min followed by 4-min elongation at 72°C. A reaction mixture without template DNA, as negative control, was included in every set of five clinical specimens for each PCR run. A sample was considered HPV-positive if one of the three amplification methods was positive, and negative if all tests were negative.

HPV DNA Sequence Analysis

HPV genotypes were identified by direct sequence analyses carried out on the single round PCR products obtained from MY09/MY11 or GP5+/GP6+ amplifications and on the nested PCR products obtained from each HPV-positive sample. PCR amplification products were extracted with phenol and chloroform-isoamyl alcohol and purified by precipitation at 37°C for 15 min in 1.25 M NaCl with 10% or 20% polyethylene glycol (PEG 6000) depending on DNA size being >300 bp or >100 bp, respectively [Tornesello et al., 2000]. Purified DNA samples were subjected to direct nucleotide sequencing using a rapid method modified from Winship [1989]. Briefly DNA samples (30 ng to 100 ng) were denatured at 95°C, in presence of 10% DMSO, immediately cooled in liquid nitrogen and subsequently sequenced with the Sequenase 2.0 kit according to manufacturer's instructions (GE Healthcare, Buckinghamshire, UK) modified in the labeling step (3 min on ice). Nine samples showing faint band PCR-amplification products were subjected to direct sequence using AmpliCycle sequencing kit following the manufacturer's protocol (of AmpliTaq DNA polymerase (Applera), specific for limited quantity (0.5 ng to 30 ng) of PCR products. HPV type identification was carried out by alignments of HPV sequences with those present in the GenBank database using the BLASTn software (http://www.ncbi.nlm.nih.gov/blast/html).

Statistical Analysis

The data were analyzed using the χ^2 test and, where appropriate, Fisher's exact test to calculate all *P*-values related to the differences between groups with Epi Info 6 Statistical Analysis System Software (6.04b, 1997, Centers for Disease Control and Prevention, USA). To assess the risk of cervical neoplasia associated with HPV types, odds ratio and 95% confidence intervals were estimated using as reference category subjects with HPV negative results. Differences were considered to be statistically significant when *P*-values were less than 0.05.

RESULTS

This study included 251 patients, with clinical presentations compatible with cervical neoplastic lesions, and 207 control subjects. Following cytological/histological typing and DNA quality/quantity assays, 183 samples were confirmed as neoplasia-free control samples, 101 were classified as low-grade squamous intraepithelial lesions, 65 as high-grade squamous intraepithelial lesions, and 65 as invasive cervical squamous carcinomas. The median age was 40.05 years (mean 41.04 ± 9.74) for control women, 35.05 years (mean 35.00 ± 7.43) for women with low-grade squamous intraepithelial lesions, 38.00 years (mean 36.87 ± 8.28) for women with high-grade squamous intraepithelial lesions, and 53.5 years (mean 55.04 ± 14.9) for women with cervical squamous cell carcinomas.

All samples were examined for HPV DNA using three methods: (1) single round PCR amplification using MY09/MY11 primer pairs [Resnick et al., 1990]; (2) single round PCR amplification using GP5+/GP6 primer pairs [de Roda Husman et al., 1995]; and (3) nested PCR amplification using MY09/MY11 (outer oligoprimers) followed by GP5+/GP6+ (inner primers). The overall HPV positivity, assessed by one or more methods, was of 19.7% (36/183) among controls, 63.4% (64/101) in low-grade squamous intraepithelial lesions, 80% (52/65) in high-grade squamous intraepithelial lesions, and 81.5% (53/65) in invasive cervical squamous carcinomas. As shown in Table I, the nested PCR method was the most sensitive ($P < 0.001$) with average positive rate of 99.5% compared to 77.6% of the MY09/MY11 and to 89.3% of the GP5+/GP6+ methods.

Direct sequence analysis undertaken on the amplification products, obtained by one of the two single round amplification methods and from the nested PCR for each sample, allowed the identification of 31 mucosotropic HPV types among the 205 HPV-positive samples, of which 16 types had been previously epidemiological classified as high-risk HPV viruses (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 66, 68, 73 and 82), 10 types as low-risk (HPV6, 11, 42, 54, 61, 70, 71, 72, 81, 83), and 5 types (HPV30, 32, 62, 67, and 90) belong to the group of undetermined-risk HPV types. Among all the HPVs identified, a statistically significant increase in the positive rate as shown by nested PCR amplification versus single round MY09/MY11 amplification was observed for HPV16 ($P < 0.05$). The MY09/MY11 method failed to detect HPV30, 32, 35, 39, 42, 67, 70, 90 and the double infections HPV16/11 (amplification of only HPV11) and HPV45/73 (amplification of only HPV45), while the GP5+/GP6+ failed to amplify the HPV54, 61, 68 and double infection HPV58/6 (amplification of only HPV6). Both methods did not amplified HPV30 and HPV70 viruses. The nested PCR method showed a significantly higher detection rate for all HPV types ($P < 0.001$).

HPV16 was viral genotype identified most commonly in all neoplastic and non-neoplastic conditions being present in 8.7% of controls, 36.6% of low-grade, and 46.1% of high-grade squamous intraepithelial lesions, respectively, and in 58.5% of invasive cervical squamous carcinomas. Common types other than HPV16 were HPV18 identified in 6.1% of high-grade squamous intraepithelial lesions and in 6.1% of invasive cervical squamous carcinomas; HPV33 in 3.1% of high-grade squamous intraepithelial lesions and in 6.1% of invasive cervical squamous carcinomas; HPV31 present in 3% of low-grade squamous intraepithelial lesions, in 3.1% of high-grade squamous intraepithelial lesions and in 4.6% of invasive cervical squamous carcinomas; HPV53 identified in 4.6% of high-grade squamous intraepithelial lesions; HPV58 and HPV81 identified each in 3.1% of high-grade squamous intraepithelial lesions. Other HPV types occurring at a frequency of less than 3.0% in high-grade squamous intraepithelial lesions and/or invasive cervical squamous carcinomas

TABLE I. Detection Rate of HPV Types in 205 HPV-Positive Samples With Three Different Amplification Protocols

HPV type	MY09/MY11 n (%)	GP5+/GP6+ n (%)	MY09/MY11 & GP5+/GP6+ n (%)	Total[a] n[b] (%)
HPV 6	4 (1.9)	3 (1.5)	4 (1.9)	4 (1.9)
HPV11	2 (1.0)	3 (1.5)	3 (1.5)	3 (1.5)
HPV16	99 (48.3)	117 (57.0)	120 (58.5)	121 (59.0)
HPV18	8 (3.9)	8 (3.9)	8 (3.9)	8 (3.9)
HPV30	0	0	1 (0.5)	1 (0.5)
HPV31	7 (3.4)	9 (4.4)	9 (4.4)	9 (4.4)
HPV32	0	1 (0.5)	1 (0.5)	1 (0.5)
HPV33	7 (3.4)	8 (3.9)	8 (3.9)	8 (3.9)
HPV35	0	1 (0.5)	1 (0.5)	1 (0.5)
HPV39	0	1 (0.5)	1 (0.5)	1 (0.5)
HPV42	0	1 (0.5)	1 (0.5)	1 (0.5)
HPV45	2 (1.0)	2 (1.0)	2 (1.0)	2 (1.0)
HPV51	1 (0.5)	1 (0.5)	2 (1.0)	2 (1.0)
HPV52	2 (1.0)	1 (0.5)	2 (1.0)	2 (1.0)
HPV53	4 (1.9)	2 (1.0)	6 (2.9)	6 (2.9)
HPV54	2 (1.0)	0	2 (1.0)	2 (1.0)
HPV56	2 (1.0)	2 (1.0)	3 (1.5)	3 (1.5)
HPV58	4 (1.9)	2 (1.0)	4 (1.9)	4 (1.9)
HPV61	1 (0.5)	0	1 (0.5)	1 (0.5)
HPV62	1 (0.5)	2 (1.0)	2 (1.0)	2 (1.0)
HPV66	2 (1.0)	5 (2.4)	5 (2.4)	5 (2.4)
HPV67	0	1 (0.5)	1 (0.5)	1 (0.5)
HPV68	1 (0.5)	0	1 (0.5)	1 (0.5)
HPV70	0	0	1 (0.5)	1 (0.5)
HPV71	1 (0.5)	1 (0.5)	1 (0.5)	1 (0.5)
HPV72	1 (0.5)	1 (0.5)	1 (0.5)	1 (0.5)
HPV73	1 (0.5)	1 (0.5)	2 (1.0)	2 (1.0)
HPV81	3 (1.5)	3 (1.5)	3 (1.5)	3 (1.5)
HPV82	1 (0.5)	2 (1.0)	2 (1.0)	2 (1.0)
HPV83	1 (0.5)	1 (0.5)	1 (0.5)	1 (0.5)
HPV90	0	1 (0.5)	1 (0.5)	1 (0.5)
HPV16/11	0	1 (0.5)	1 (0.5)	1 (0.5)
HPV16/73	1 (0.5)	1 (0.5)	1 (0.5)	1 (0.5)
HPV45/73	0	1 (0.5)	1 (0.5)	1 (0.5)
HPV58/6	1 (0.5)	0	1 (0.5)	1 (0.5)
Any HPV	159[b] (77.6)	183[b] (89.3)	204 (99.5)	205 (100)

[a]Results obtained by the combination of the three methods.
[b]P-value <0.001, Yates corrected χ^2 test.

were HPV35, 54, 56, 62, 70, 72, 82, and 90. All the remaining HPV genotypes (HPV6, 11, 30, 32, 51, 39, 42, 45, 51, 52, 61, 66, 67, 68, 73, and 83) were detected only in controls and low-grade squamous intraepithelial lesions. Double infections were infrequent being present in 2% of low-grade squamous intraepithelial lesions (HPV45/73 and HPV58/6), in 1.5% of high-grade squamous intraepithelial lesions (HPV16/11), and 1.5% of invasive cervical squamous carcinomas (HPV16/73).

Overall, the high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 66, 68, 73, and 82) were identified in 16.9% of controls, in 50.5% of low-grade squamous intraepithelial lesions, in 69.2 of high-grade squamous intraepithelial lesions, and in 78.5% of invasive cervical squamous carcinomas, while the low-risk HPV types (HPV6, 11, 42, 54, 61, 70, 71, 72, 81, 83) were present in only 2.7% of controls, 6.4% of low-grade squamous intraepithelial lesions, 6.1% of high-grade squamous intraepithelial lesions. Five HPV genotypes whose epidemiological risk has not yet been assessed have been identified in 3% of low-grade squamous intraepithelial lesions (HPV32, 30, 67), in 3.1% of high-grade squamous intraepithelial lesions (HPV62 and 90), and in 1.5% of invasive cervical squamous carcinomas (HPV62) but in none of the 187 controls.

The prevalence of HPV DNA among patients and controls, and the corresponding odds ratios are presented in Table II according to disease stage. All high-grade squamous intraepithelial lesions and invasive cervical squamous carcinomas patients had significantly higher prevalence of infection with any HPV (OR = 16.3, 95%CI, 7.6–35.4 for high-grade squamous intraepithelial lesions; OR = 18.0, 95%CI, 8.3–39.9 for invasive cervical squamous carcinomas) and with high-risk HPVs (OR = 16.4, 95%CI, 7.5–36.5 for high-grade squamous intraepithelial lesions; OR = 20.1, 95%CI, 9.1–95.4 for invasive cervical squamous carcinomas), but not with low-risk viruses, when compared to HPV negative controls. Low-grade squamous intraepithelial lesion patients had a significantly higher prevalence of both high-risk (OR = 7.1, 95%CI, 3.9–12.7) and low-risk

TABLE II. HPV Genotype Distribution Among Different Cytological/Histological Groups: Controls, Low-Grade (LSIL), and High-Grade Squamous Intraepithelial Lesions (HSIL), and Invasive Cervical Carcinomas (ICC)

	HPV	HPV−positive cytological/histological groups						
Species	Type	Controls (%) (n = 183)	LSIL (%) (n = 101)	Odds ratio (95%CI)	HSIL (%) (n = 65)	Odds ratio (95%CI)	ICC (%) (n = 65)	Odds ratio (95%CI)
	HPV negative	147 (80.3)	37 (36.6)		13 (20.0)		12 (18.5)	
	HPV positive	36 (19.7)	64 (63.4)	7.1 (3.9–12.7)	52 (80.0)	16.3 (7.6–35.4)	53 (81.5)	18.0 (8.3–39.9)
5	HPV51	1 (0.5)	1 (1.0)	4.0 (0.0–149.4)	0	0	0	0
5	HPV82	1 (0.5)	0	0	0	0	1 (1.5)	12.2 (0.0–486.1)
6	HPV53	1 (0.5)	2 (2.0)	7.9 (0.5–227.9)	3 (4.6)	33.9 (2.8–914.4)	0	0
6	HPV56	2 (1.1)	0	0	1 (1.5)	0	0	0
6	HPV66	3 (1.6)	2 (2.0)	2.6 (0.3–20.5)	0	0	0	0
7	HPV18	0	0	0	4 (6.1)	0	4 (6.1)	0
7	HPV39	1 (0.5)	0	0	0	0	0	0
7	HPV45	1 (0.5)	1 (1.0)	4.0 (0.0–149.4)	0	0	0	0
7	HPV68	0	1 (1.0)	0	0	0	0	0
7	HPV70	0	0	0	1 (1.5)	0	0	0
9	HPV16	16 (8.7)	37 (36.6)	9.2 (4.4–19.4)	30 (46.1)	21.2 (8.6–53.4)	38 (58.5)	29.1 (11.8–73.3)
9	HPV31	1 (0.5)	3 (3.0)	11.9 (1.1–306.4)	2 (3.1)	22.6 (1.4–680.1)	3 (4.6)	36.7 (3.0–996.8)
9	HPV33	0	2 (2.0)	0	2 (3.1)	0	4 (6.1)	0
9	HPV35	0	0	0	0	0	1 (1.5)	0
9	HPV52	1 (0.5)	1 (1.0)	4.0 (0.0–149.4)	0	0	0	0
9	HPV58	1 (0.5)	1 (1.0)	4.0 (0.0–149.4)	2 (3.1)	22.6 (1.4–680.2)	0	0
11	HPV73	2 (1.1)	0	0	0	0	0	0
Total high risk[a]		31 (16.9)	51 (50.5)	6.5 (3.5–12.1)	45 (69.2)	16.4 (7.5–36.5)	51 (78.5)	20.1 (9.1–45.4)
1	HPV42	0	1 (1.0)	0	0	0	0	0
3	HPV81	0	1 (1.0)	0	2 (3.1)	0	0	0
3	HPV61	0	1 (1.0)	0	0	0	0	0
3	HPV72	0	0	0	1 (1.5)	0	0	0
3	HPV83	1 (0.5)	0	0	0	0	0	0
10	HPV6	3 (1.6)	1 (1.0)	1.3 (0.0–17.1)	0	0	0	0
10	HPV11	1 (0.5)	2 (2.0)	7.9 (0.5–227.9)	0	0	0	0
13	HPV54	0	1 (1.0)	0	1 (1.5)	0	0	0
15	HPV71	0	1 (1.0)	0	0	0	0	0
Total low risk[a]		5 (2.7)	8 (7.9)	6.4 (1.7– 24.0)	4 (6.1)	0	0	0
1	HPV32	0	1 (1.0)	0	0	0	0	0
3	HPV62	0	0	0	1 (1.5)	0	1 (1.5)	0
6	HPV30	0	1 (1.0)	0	0	0	0	0
9	HPV67	0	1 (1.0)	0	0	0	0	0
14	HPV90	0	0	0	1 (1.5)	0	0	0
Total unknown risk		0	3 (3.0)	0	2 (3.1)	0	1 (1.5)	0
9, 10	HPV16/11	0	0	0	1 (1.5)	0	0	0
9, 11	HPV16/73	0	0	0	0	0	1 (1.5)	0
7, 11	HPV45/73	0	1 (1.0)	0	0	0	0	0
9, 1	HPV58/6	0	1 (1.0)	0	0	0	0	0
Total multiple infections		0	2 (2.0)	0	1 (1.5)	0	1 (1.5)	0

[a]Low− and high-risk grouping following epidemiologic and/or phylogenetic classification as defined by Munoz et al., 2003.

(OR = 6.4, 95%CI, 1.7–24.0) viruses versus controls. HPV types detected only in low-grade squamous intraepithelial lesions (HPV30, 32, and 67), or in high-grade squamous intraepithelial lesions and invasive cervical squamous carcinomas (HPV62 and 90) but not detected in no control samples should be designated "probable high-risk" and "high-risk" viruses, respectively, and included in the panel of HPV types used for wide population screening.

Figure 1 shows the analysis of all HPV isolates grouped in their corresponding species, as percentage of each HPV species among all HPV-positive samples for each pathology group, assuming that HPVs related phylogenetically have presumably similar biochemical and biological properties, including in some extent neutralizing antibodies cross-reactivity. The species 9, comprising HPV16 and related types 31, 33, 35, 52, and 58, was the most frequent in controls (52.8%), in low-grade squamous intraepithelial lesions (68.8%), in high-grade squamous intraepithelial lesions (69.2%), and in invasive cervical squamous carcinomas (86.8%). The next most common was species 7, comprising the HPV18 and 39, 45, 68, and 70, in low-grade squamous intraepithelial lesions (4.7%), in high-grade squamous intraepithelial lesions (9.7%), and invasive cervical squamous carcinomas (7.5%); and species 6 (HPV53, 56, 66) in controls (30%), in low-grade squamous intraepithelial lesions (7.8%), in high-grade squamous intraepithelial lesions (7.7%) but not in invasive cervical squamous carcinomas.

DISCUSSION

The prevalence of type specific was examined in normal cervical scrapes, in low-grade, in high-grade neoplastic lesions and in cervical carcinomas from

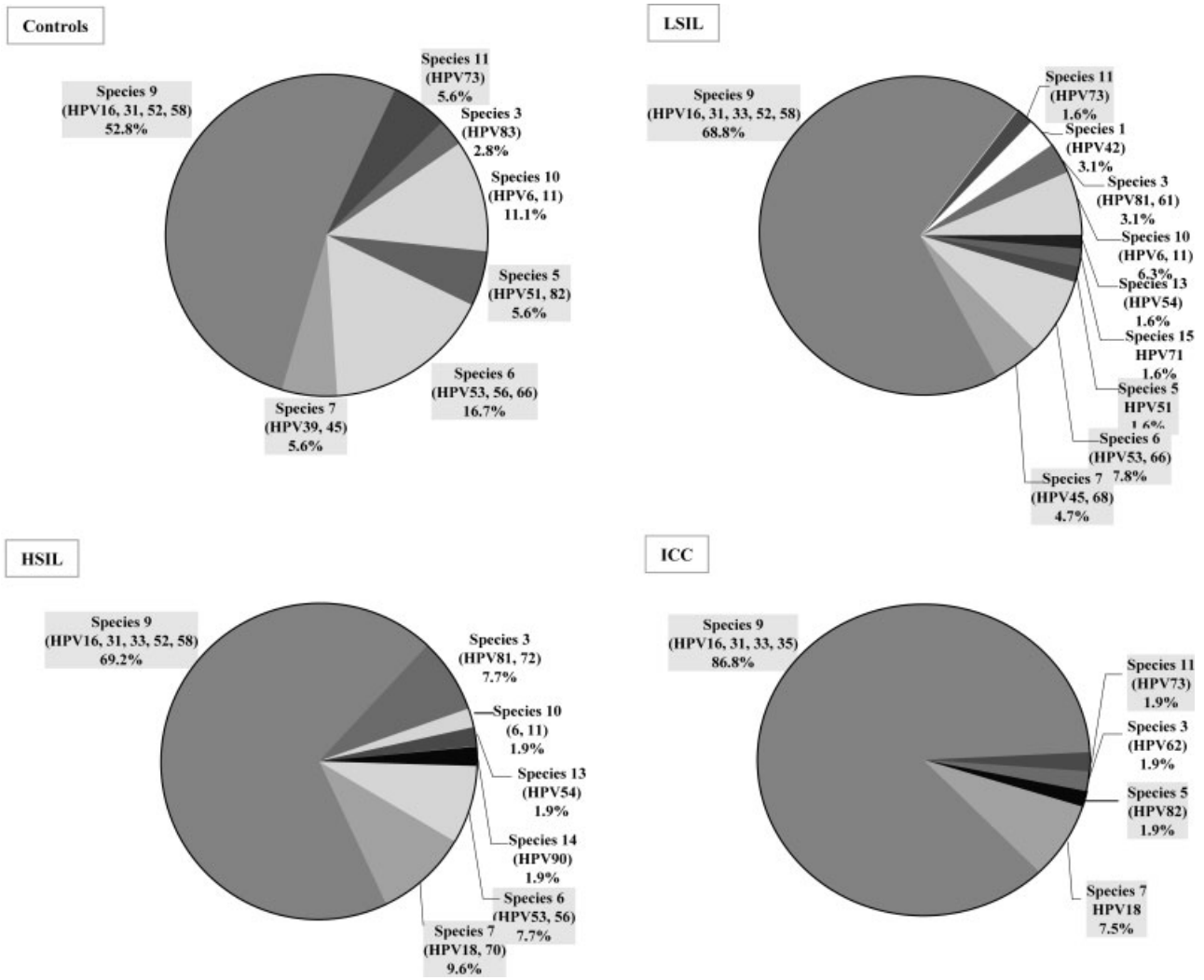

Fig. 1. Distribution in controls, in low-grade (LSIL) and high-grade (HSIL) squamous intraepithelial lesions, and cervical carcinoma (ICC) samples of 31 HPV viral genotypes, grouped in 11 alfa species. Representativeness of each species is expressed as percentage of all HPV positive cases in each category.

Italian Caucasian women living in Northern and Southern Italy. Viral infections have been detected with the most commonly used MY09/MY11 and GP5+/GP6+ primer pairs, by three different protocols, and have been genotyped by direct sequencing analysis. There was a differential amplification of HPV types between the MY09/MY11 and GP5+/GP6+ single round amplification methods. The most marked difference was seen in the ability of MY09/MY11 to amplify HPV16 DNA compared to GP5+/GP6+ primer pairs ($P = 0.07$). The poor efficacy of MY09/MY11 to amplify HPV35 and of GP5+/GP6+ to amplify HPV53 and 61 is in accordance with previous studies [Qu et al., 1997; Chan et al., 2006]. The MY09/MY11 failed to amplify HPV39, 42, 67, 70, and 90 genotypes, while GP5+/GP6+ failed to amplify HPV54 and 68. The nested PCR method carried out with MY09/MY11, as outer primer pairs, and GP5+/GP6+, as inner primer pairs, was significantly more sensitive ($P < 0.001$) for detection of all HPV genotypes identified in this study and allowed the identification of HPV30 and 70, not detected by either MY09/MY11 nor GP5+/GP6+ alone.

Overall 31 viral genotypes, grouped in 11 HPV species, were identified including 16 epidemiological defined high oncogenic risk types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 66, 68, 73, and 82) and 10 low oncogenic risk types (HPV6, 11, 42, 54, 61, 70, 71, 72, 81, 83). As expected, the total number of different HPV types identified was higher in low-grade squamous intraepithelial lesions (20 genotypes) and high-grade squamous intraepithelial lesions (14 genotypes) than in cervical carcinomas (8 genotypes) ($P < 0.005$). Moreover, considering only the number of different high-risk HPV genotypes in controls (12 HPVs), in low-grade squamous intraepithelial lesions (10 HPVs), in high-grade squamous intraepithelial lesions (8 HPVs) and in invasive cervical squamous carcinomas (6 HPVs) samples, it is evident that the major difference in genotype heterogeneity is due to a negative gradient of low-risk viruses from benign to malignant lesions. In agreement with prevalence rates reported previously, the HPV16 showed the highest prevalence rate of infection among all groups examined with 8.7%, 36.6%, 46.1%, and 58.5% positivity in controls, low-grade and high-grade squamous intraepithelial lesions, and invasive cervical squamous carcinomas, respectively [Zerbini et al., 2001; De Francesco et al., 2005; Ronco et al., 2005; Clifford et al., 2005b]. The HPV16 overall prevalence increase in neoplastic lesions is associated with a major higher frequency of non-European variants in advanced disease stages, suggesting their higher oncogenic activity [Tornesello et al., 2004]. The second most common types were HPV18, 31, and 33 in high-grade squamous intraepithelial lesions and invasive cervical squamous carcinomas with a prevalence ranging from 3.1 to 6.1%. Four high-risk genotypes HPV53, 56, 70, and 58, although underrepresented in cervical cancer, were present in over 10% of high-grade squamous intraepithelial lesions, highlighting a considerable potential to cancer progression. Other high-risk genotypes (HPV51, 66, 39, 45, 68, 52, and 73) have been found exclusively in controls and in low-grade squamous intraepithelial lesions, with a prevalence similar to that reported by Ronco et al. [2005] in women from Turin, suggesting a lower oncogenic potential compared to other risk types. Thus, HPV53 and 66, both previously classified as probable risk types, on the basis of their detection frequency in low-grade squamous intraepithelial lesions and in high-grade squamous intraepithelial lesions may be defined as high-risk and low-risk types, respectively. The identification of HPV82 in a cervical carcinoma sample is supportive of the high-risk classification proposed by Munoz et al. [2003]. The identification of HPV types HPV54, 72, and 81, found previously only in cytological normal subjects (3.1%)and therefore classified as low-risk types, have been detected in 3.1% of low-grade squamous intraepithelial lesions and in 6.1% of high-grade squamous intraepithelial lesions and no controls raising questions on the oncogenic potential of these uncommon viral types.

Furthermore, six viral genotypes (HPV30, 32, 62, 67, and 90), which were not included in the cocktail of HPV types used for viral detection in previous screening studies carried out in Europe [Clifford et al., 2005a], have been identified. Among these, HPV30, 32, and 67 present only in low-grade squamous intraepithelial lesions and found previously in non-neoplastic samples from Indian women [Franceschi et al., 2005] may be defined low-risk types. HPV32, however, has been detected previously in oral papillomas [de Villiers et al., 1986], in one oral focal epithelial hyperplasia [Beaudenon et al., 1987] and more recently in oral warts from HIV-positive patients, where it has been shown to selectively enhance epithelial cell growth and differentiation in the stratum spinosum [Baumgarth et al., 2004], raising questions on tissue tropism and viral pathogenesis in different mucosal sites. HPV62, first identified in normal cervical samples [Bernard et al., 1994] and in dysplastic lesions of the esophagus [Lavergne and de Villiers, 1999] has been detected both in high-grade squamous intraepithelial lesions (1 out of 65) and in invasive cervical squamous carcinomas (1 out of 65); HPV90, first isolated from a normal cervical cytological sample from Hispanic young women in USA [Terai and Burk, 2002] and later identified in a cytological normal sample from Chilean woman [Ferreccio et al., 2004], has been detected in 1 out of 65 high-grade squamous intraepithelial lesions. Thus HPV62 and HPV90 needs further analysis in larger population studies in order to define their oncogenic risk.

The low detection rate of these viral types could be due to biases in sensitivity of commonly used amplification primer pairs (i.e., HPV90 resulted not amplifiable with MY09/MY11 PCR system as reported previously by Terai and Burk, 2001), or to a narrow window of HPV transient infections. The newly introduced viral types within a given region, by population mobility such as immigration or travel, could also result in low detection rate of those HPV genotypes in the general population. Indeed, the characterization of HPV genotypes in

cytologically normal Nigerian women Immigrants in South Italy showed a high frequency of infection (>30%) with HPV types 33 (17.6%) and HPV70 (17.6%), followed by HPV35 (11.8%), HPV58 (11.8%), HPV81 (11.8%), HPV54 (5.9%), and HPV67 (5.9%), and absence of HPV16 and 18, against which most vaccine strategies have been developed [Tornesello et al., 2006].

Furthermore, serological studies evaluating the natural history of different class of antibodies production against HPV16 virus like particles (HPV16 VLP), following exposure to HPV16, to HPV16-related genotypes (species 9, comprising HPV31, 33, 35, 52, 58) and HPV16-unrelated types, showed that only HPV16 and in some extent high viral load of HPV16-related types but not other high-risk HPVs are able to induce antibodies cross-reactive against HPV16-based VLPs [Ho et al., 2002].

In conclusion, HPV16 and HPV-16-related types were the most prevalent types in all degree of malignancies among Italian women. Moreover HPVs other than type 16 showed an heterogeneous type distribution particularly in low-grade and high-grade squamous intraepithelial lesions not reported by previous studies. The presence of uncommon high-risk HPVs and their role in the pathogenesis of cervical cancer need to be addressed by further epidemiological and molecular studies. The surveillance of risk groups that may act as viral reservoirs within different countries, are necessary to determine the efficacy of current vaccines and mandatory to design future vaccine strategies and screening tests.

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REFERENCES

Baumgarth N, Szubin R, Dolganov GM, Watnik MR, Greenspan D, Da Costa M, Palefsky JM, Jordan R, Roederer M, Greenspan JS. 2004. Highly tissue substructure-specific effects of human papilloma virus in mucosa of HIV-infected patients revealed by laser-dissection microscopy-assisted gene expression profiling. Am J Pathol 165:707–718.

Beaudenon S, Praetorius F, Kremsdorf D, Lutzner M, Worsaae N, Pehau-Arnaudet G, Orth G. 1987. A new type of human papillomavirus associated with oral focal epithelial hyperplasia. J Invest Dermatol 88:130–135.

Bernard HU, Chan SY, Manos MM, Ong CK, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM. 1994. Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. J Infect Dis 170: 1077–1085.

Bosch FX, de Sanjose S. 2003. Chapter 1: Human papillomavirus and cervical cancer–burden and assessment of causality. J Natl Cancer Inst Monogr. 31:3–13.

Buonaguro FM, Tornesello ML, Beth-Giraldo E, Hatzakis A, Mueller N, Downing R, Biryamwaho B, Sempala SD, Giraldo G. 1996. Herpesvirus-like DNA sequences detected in endemic, classic, iatrogenic and epidemic Kaposi's sarcoma (KS) biopsies. Int J Cancer 65:25–28.

Buonaguro FM, Tornesello ML, Salatiello I, Okong P, Buonaguro L, Beth-Giraldo E, Biryahwaho B, Sempala SD, Giraldo G. 2000. The Uganda study on HPV variants and genital cancers. J Clin Virol 19: 31–41.

Chan PK, Cheung TH, Tam AO, Lo KW, Yim SF, Yu MM, To KF, Wong YF, Cheung JL, Chan DP, Hui M, Ip M. 2006. Biases in human papillomavirus genotype prevalence assessment associated with commonly used consensus primers. Int J Cancer 118:243–245.

Clifford GM, Smith JS, Aguado T, Franceschi S. 2003a. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: A meta-analysis. Br J Cancer 89:101–105.

Clifford GM, Smith JS, Plummer M, Munoz N, Franceschi S. 2003b. Human papillomavirus types in invasive cervical cancer worldwide: A meta-analysis. Br J Cancer 88:63–73.

Clifford GM, Gallus S, Herrero R, Munoz N, Snijders PJ, Vaccarella S, Anh PT, Ferreccio C, Hieu NT, Matos E, Molano M, Rajkumar R, Ronco G, de Sanjose S, Shin HR, Sukvirach S, Thomas JO, Tunsakul S, Meijer CJ, Franceschi S; IARC HPV Prevalence Surveys Study Group. 2005a. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: A pooled analysis. Lancet 366:991–998.

Clifford GM, Rana RK, Franceschi S, Smith JS, Gough G, Pimenta JM. 2005b. Human papillomavirus genotype distribution in low-grade cervical lesions: Comparison by geographic region and with cervical cancer. Cancer Epidemiol Biomarkers Prev 14:1157–1164.

De Francesco MA, Gargiulo F, Schreiber C, Ciravolo G, Salinaro F, Manca N. 2005. Detection and genotyping of human papillomavirus in cervical samples from Italian patients. J Med Virol 75:588–592.

de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. 1995. The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 76: 1057–1062.

de Villiers EM, Neumann C, Le JY, Weidauer H, zur Hausen H. 1986. Infection of the oral mucosa with defined types of human papillomaviruses. Med Microbiol Immunol (Berl) 174:287–294.

de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. 2004. Classification of papillomaviruses. Virology 324:17–27.

Ferreccio C, Prado RB, Luzoro AV, Ampuero SL, Snijders PJ, Meijer CJ, Vaccarella SV, Jara AT, Puschel KI, Robles SC, Herrero R, Franceschi SF, Ojeda JM. 2004. Related Population-based prevalence and age distribution of human papillomavirus among women in Santiago, Chile. Cancer Epidemiol Biomarkers Prev 13: 2271–2276.

Franceschi S, Rajkumar R, Snijders PJ, Arslan A, Mahe C, Plummer M, Sankaranarayanan R, Cherian J, Meijer CJ, Weiderpass E. 2005. Papillomavirus infection in rural women in southern India. Br J Cancer 92:601–606.

Fredricks DN, Relman DA. 1999. Paraffin removal from tissue sections for digestion and PCR analysis. Biotechniques 26:198–200.

Harper DM, Franco EL, Wheeler C, Ferris DG, Jenkins D, Schuind A, Zahaf T, Innis B, Naud P, De Carvalho NS, Roteli-Martins CM, Teixeira J, Blatter MM, Korn AP, Quint W, Dubin G; GlaxoSmithKline HPV Vaccine Study Group. 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: A randomised controlled trial. Lancet 364:1757–1765.

Harper DM, Franco EL, Wheeler CM, Moscicki AB, Romanowski B, Roteli-Martins CM, Jenkins D, Schuind A, Costa Clemens SA, Dubin G; HPV Vaccine Study group. 2006. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: Follow-up from a randomised control trial. Lancet 367:1247–1255.

Ho GY, Studentsov Y, Hall CB, Bierman R, Beardsley L, Lempa M, Burk RD. 2002. Risk factors for subsequent cervicovaginal human papillomavirus (HPV) infection and the protective role of antibodies to HPV-16 virus-like particles. J Infect Dis 186:737–742.

Ho GY, Studentsov YY, Bierman R, Burk RD. 2004. Natural history of human papillomavirus type 16 virus-like particle antibodies in young women. Cancer Epidemiol Biomarkers Prev 13:110–116.

Husnjak K, Grce M, Magdic L, Pavelic K. 2000. Comparison of five different polymerase chain reaction methods for detection of human papillomavirus in cervical cell specimens. J Virol Methods. 88:125–134.

Kirnbauer R, Hubbert NL, Wheeler CM, Becker TM, Lowy DR, Schiller JT. 1994. A virus-like particle enzyme-linked immunosorbent assay

detects serum antibodies in a majority of women infected with human papillomavirus type 16. J Natl Cancer Inst 86:494–499.

Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, Chiacchierini LM, Jansen KU; Proof of Principle Study Investigators. 2002. A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med 347:1645–1651.

Lavergne D, de Villiers EM. 1999. Papillomavirus in esophageal papillomas and carcinomas. Int J Cancer 80:681–684.

Munoz N. 2000. Human papillomavirus and cancer: The epidemiological evidence. J Clin Virol 19:1–5.

Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348:518–527.

Qu W, Jiang G, Cruz Y, Chang CJ, Ho GY, Klein RS, Burk RD. 1997. PCR detection of human papillomavirus: Comparison between MY09/MY11 and GP5+/GP6+ primer systems. J Clin Microbiol 35: 1304–1310.

Resnick RM, Cornelissen MT, Wright DK, Eichinger GH, Fox HS, ter Schegget J, Manos MM. 1990. Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. J Natl Cancer Inst 82:1477–1484.

Ronco G, Ghisetti V, Segnan N, Snijders PJ, Gillio-Tos A, Meijer CJ, Merletti F, Franceschi S. 2005. Prevalence of human papillomavirus infection in women in Turin, Italy. Eur J Cancer 41:297–305.

Sasagawa T, Yamazaki H, Dong YZ, Satake S, Tateno M, Inoue M. 1998. Immunoglobulin-A and -G responses against virus-like particles (VLP) of human papillomavirus type 16 in women with cervical cancer and cervical intra-epithelial lesions. Int J Cancer 75: 529–535.

Terai M, Burk RD. 2001. Characterization of a novel genital human papillomavirus by overlapping PCR: Candhpv86 identified in cervicovaginal cells of a woman with cervical neoplasia. J Gen Virol 82:2035–2040.

Terai M, Burk RD. 2002. Identification and characterization of 3 novel genital human papillomaviruses by overlapping polymerase chain reaction: Candhpv89, candHPV90, and candHPV91. J Infect Dis 185:1794–1797.

Tornesello ML, Buonaguro FM, Meglio A, Buonaguro L, Beth-Giraldo E, Giraldo G. 1997. Sequence variations and viral genomic state of human papillomavirus type 16 in penile carcinomas from Ugandan patients. J Gen Virol 78:2199–2208.

Tornesello ML, Buonaguro FM, Buonaguro L, Salatiello I, Beth-Giraldo E, Giraldo G. 2000. Identification and functional analysis of sequence rearrangements in the long control region of human papillomavirus type 16 Af-1 variants isolated from Ugandan penile carcinomas. J Gen Virol 81:2969–2982.

Tornesello ML, Duraturo ML, Salatiello I, Buonaguro L, Losito S, Botti G, Stellato G, Greggi S, Piccoli R, Pilotti S, Stefanon B, De Palo G, Franceschi S, Buonaguro FM. 2004. Analysis of human papillomavirus type-16 variants in Italian women with cervical intraepithelial neoplasia and cervical cancer. J Med Virol 74:117–126.

Tornesello ML, Duraturo ML, Buonaguro L, De Falco C, Vallefuoco G, Piccoli R, Palmieri S, Buonaguro FM. 2006. Heterogeneous distribution of human papillomavirus genotypes in high risk West Africa women immigrants in South Italy. 6th International multidisciplinary congress Eurogin 2006-Paris, April 23-26:71.

Van Ranst M, Kaplan JB, Burk RD. 1992. Phylogenetic classification of human papillomaviruses: Correlation with clinical manifestations. J Gen Virol 73:2653–2660.

Villa LL, Costa RL, Petta CA, Andrade RP, Ault KA, Giuliano AR, Wheeler CM, Koutsky LA, Malm C, Lehtinen M, Skjeldestad FE, Olsson SE, Steinwall M, Brown DR, Kurman RJ, Ronnett BM, Stoler MH, Ferenczy A, Harper DM, Tamms GM, Yu J, Lupinacci L, Railkar R, Taddeo FJ, Jansen KU, Esser MT, Sings HL, Saah AJ, Barr E. 2005. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: A randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol 6:271–278.

Winship PR. 1989. An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. Nucleic acids Res 17: 1266–1266.

Zerbini M, Venturoli S, Cricca M, Gallinella G, De Simone P, Costa S, Santini D, Musiani M. 2001. Distribution and viral load of type specific HPVs in different cervical lesions as detected by PCR-ELISA. J Clin Pathol 54:377–380.

zur Hausen H. 2000. Papillomaviruses causing cancer: Evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 92:690–698.