Prevalence of Oncogenic HPV Types in Sardinia (Italy): Implications for the Impact of Prophylactic Anti-HPV Vaccines

[1]Stefania Montisci, [1]Sabrina Pitzalis, [1]Marianna Greco, [2]Alberto Orani, [3]Marco Rais and [1]Sergio Laconi
[1]Department of Biomedical Sciences and Biotechnology, University of Cagliari, Cagliari 09124, Italy.
[2]Centro di Prevenzione Oncologica, ASL 8, Cagliari, Italy.
[3]Unità di Anatomia Patologica, Ospedale Oncologico A. Businco, ASL 8, Cagliari, Italy

Abstract: Infection with high-risk HPV genotypes is considered an essential step in cervical carcinogenesis. Recently, prophylactic anti-HPV vaccines have shown to provide effective protection in clinical trials. However, protection appears to be type-restricted, thus, its real extent will depend on HPV types prevalence in the target population. Here we report HPV prevalence in 555 Italian women with various stages of HPV-related cervical disease (343 CIN1/L-SIL, 156 CIN2-3/HIGH-SIL, 43 invasive squamous cervical carcinoma and 13 adenocarcinoma), as well as in 315 women with smears negative for intraepithelial lesions or malignancy (NIL). HPV was found in 50.7% of CIN1/L-SIL, 87.2% of CIN2-3/HIGH-SIL, 97.7% of squamous carcinomas, 69.2% of adenocarcinomas and in 29.5% of NIL. HPV 16 was present in 69.0% of squamous carcinomas, 58.8% of CIN2-3/HIGH-SIL, in 22.9 and 16.1% of CIN1/L-SIL and NIL, respectively. HPV 18 was found in 7.3% and 4.8%, respectively, while in 35.3% and 26.2% of CIN2-3/HIGH-SIL and invasive carcinomas, respectively, were present high-risk HPV types other than type 16 and 18. Based on these data, it is expected that current HPV prophylactic vaccines could effectively prevent up to 70% of invasive cervical cancers and a slightly smaller proportion of high-grade lesions, in our population.

Keywords: HPV prevalence; HPV typing; High-grade lesions; Cervical cancer,

INTRODUCTION

It is well known that infection with high-risk Human Papilloma Viruses (HPVs) is a necessary step through the development of cervical cancer [1-3]. HPVs represent a large group of closely related, ubiquitary viruses that infect the skin and squamous epithelia throughout the body [4-6]. More than 100 types (genotypes) have been identified so far and new types, subtypes and variants continue to be added to the list. The so called anogenital HPVs infect preferentially the epithelial lining of the anogenital tract in both males and females, causing a wide range of proliferative lesions spanning from condilomata acuminata (genital warts) to cervical and anal cancer [5]. About 40 anogenital HPV types have been described, distinguished as either high-risk or low-risk, based on the frequency of their association with invasive cervical cancer [7-10]. Among the approximately 20 different high-risk HPV genotypes the most common worldwide are HPV 16, and HPV 18 [7-10].

HPV genotypes are identified according to the sequence of the L1 gene, which encodes for the major capsid protein of HPV. Type-specific neutralizing antibodies are produced against this protein during natural infection, but such a humoral response is thought to play little or no role in virus clearance [11, 12]. On the other hand, it is commonly accepted that neutralizing antibodies against L1 offer protection against new infections by the same HPV type, but, being type restricted, they are considered to be largely ineffective against other HPV types. The humoral response is also thought to play no role in the regression of pre-invasive lesions and in advanced stages of cervical disease. For this reason, two approaches have been taken in the development of vaccine strategies against HPV: The first approach (prophylactic) is aimed to eliciting a powerful humoral response against the same antigen(s) (the L1 protein) that trigger(s) the antibody response to natural infection; the second approach (therapeutic) is aimed to the eradication of established HPV infections through the induction of a cell-mediated immune

Corresponding Author: Sergio Laconi, MD. Ph.D., Department of Biomedical Sciences and Biotechnology, Section of Experimental Pathology, University of Cagliari, Cagliari 09124, Italy. Phone: +39 070 6758430; Fax: 39-070-662574

response against the transforming viral proteins E6 and/or E7. While some success has been achieved in the regression of transplanted cervical carcinomas in laboratory animals [13], the development of E6/E7-based, therapeutic HPV vaccines has not reached the clinical trial stage yet.

Conversely, L1-based prophylactic vaccines have proved to be highly effective in clinical trials [14]. One such vaccine, Merk's Gardasil, targets the two most prevalent high-risk HPV types (16 and 18), but also two low-risk types, HPV 6 and 11, the main viral types involved in the development of genital warts. Gardasil has recently obtained U.S. FDA approval for human use, while another vaccine developed by Smith-Kline Glaxo (Cervarix), targeted against HPV 16 and 18, is expected to follow soon. Both vaccines were able to induce high-titer, type-specific antibody responses (much higher than those obtained after natural infection) in clinical trials and they offered 100% protection against infection by either HPV 16 or 18 [15-17]. It is expected that these vaccines will have a dramatic impact on the incidence of invasive cervical cancer as well as pre-invasive cervical lesions [14, 18,19]. However, due to the long latency of cervical cancer, the decline in the incidence of this disease will not be apparent until 3 or 4 decades after the start of the immunization programs. Moreover, since, as compared to invasive cancer, a larger proportion of pre-invasive lesion may be caused by HPV types other than HPV 16 and 18 [20-21] it is predicted that the impact of vaccination on the incidence of these lesions will be less prominent.

The prevalence of the various HPV types varies among different populations and geographical areas [7, 10]. Although the reasons for such regional differences are not completely understood, due to the type-restriction of the immune response against HPV, it is evident that any accurate prediction on the efficacy of vaccines in at-risk populations has to be based on the local HPV type prevalence data. In this study we have assessed the prevalence of a panel of 25 HPV genotypes on a population of Italian women with various stages of pre-invasive cervical lesions or invasive cancer.

MATERIALS AND METHODS

Sample collection: Eight hundred-fifty one cervical samples were collected between 2001 and 2004 at the Unit of Anatomic Pathology of the Oncology Hospital "A. Businco" and the Center for Tumor Prevention in

Cagliari, Italy from women with various cytological alterations, after informed written consent. Samples consisted of either exfoliated cervical cells or cervical biopsies. All cases were re-evaluated by an expert pathologist (M.R.) and classified according to Bethesda 2001 as follows: 325 cases displayed only inflammatory or non specific changes (reactive changes, metaplasia) with no signs of intraepithelial lesions or malignancy and were classified as NIL; 359 cases showed evident signs of HPV infection (koilocytosis) and/or mild atypia and were classified as either cervical intraepithelial neoplasia (CIN) grade 1, or low-grade squamous intraepithelial lesion (L-SIL); 167 cases showed signs of severe atypia or carcinoma in situ (CIS) and were classified as CIN grade 2-3 (CIN2-3), high-grade SIL (H-SIL) or CIS.

Table 1: Women enrolled in the study

Cytological/histological diagnosis	Number	Mean Age (Range)
NIL	315	37.1 (18-67)
CIN1/L-SIL	343	34.8 (18-66)
CIN2-3/CIS/H-SIL/	156	38.8 (19-64)
SCC	43	51.2 (27-86)
AC	13	58.4 (39-76)
Total	870	

NIL = no intraepithelial neoplasia
CIN = cervical intraepithelial neoplasia
SIL = squamous intraepithelial lesion
CIS = carcinoma in situ; SCC = squamous cell carcinoma
AC = adenocarcinoma

In total, out of 922 samples, 52 did not yield any amplifiable DNA and were excluded from the study. The remaining 870 samples consisted of 315 NIL, 343 CIN1/L-SIL, 156 CIN2-3/H-SIL/CIS, 43 SCCs and 13 ACs. A list of samples used in the present study is reported in Table 1. Sixty-five samples of cell scrapings or biopsies from condilomata acuminata (genital warts) of external genitals or the perianal region, obtained after informed written consent from both female (19) and male (46) patients treated at the Division of Dermatology of the Oncology Hospital "A. Businco" in Cagliari, were also included in this study.

Sample processing and PCR: Samples were digested with a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5%Tween 20 and 2mg/ml proteinase K, for 12-16 hours at 55°C. An aliquot of crude lysate was used for the PCR. Quality of lysates was tested by PCR amplification of the beta globin gene with the following primers: forward 5'-CTTTCAGGGCAATAATGA-3', reverse 5'-TGGTAGCTGGATTGTAGC. Crude lysates that did not yield a beta globin product were extracted with phenol/chloroform and the extracted DNA re-amplified with the same beta globin primers. Samples that remained negative for beta globin were considered not amplifiable and excluded from this study.

HPV genotyping was done by PCR-reverse hybridization as previously described [22]. Briefly, a segment of the L1 region was amplified with GP5+/GP6+ consensus primers [23] and labeled during PCR through the incorporation of Dig-11-dUTP (Roche Applied Science, Mannheim, Germany). Labeled amplicons were then hybridized to a panel of 25 type specific probes (High-risk: HPV 16, 18, 26, 30, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68 and 73; low-risk: HPV 6, 11, 34, 40, 42, 43, 44, 54 and 70), previously immobilized to the surface of NucleoLink wells (NUNC, Denmark) and detected with a POD-conjugated anti-Digoxigenin antibody (Roche Applied Science, Mannheim, Germany) and the tetra methyl benzidine (TMB) substrate (Sigma-Aldrich, Milan Italy).

RESULTS

Prevalence of HPV DNA in cervical samples: We first screened all samples with consensus primer-PCR as described in methods, in order to detect the presence of HPV L1 sequences. Overall, as reported in Table 2, among the 870 samples that yielded a product for the control gene, HPV DNA was found in 52.2%. However, as expected, the prevalence of HPV varied greatly according to the severity of cellular atypia. HPV was found in 29.5% of NIL samples while more than 50% (50.7%) of low-grade lesions were found positive for HPV. This percentage increased to 87.2% in high-grade lesions and to 97.7% in SCC. By contrast less than 70% (69.2) of ACs resulted positive for HPV.

We then performed type-specific HPV genotyping by reverse hybridization to single DNA probes specific for

Table 2: Prevalence of HPV DNA in cervical samples

	HPV DNA		
	Positive	Negative	Total
NIL	93 (29.5)	222 (70.5)	315
CIN1/L-SIL	174 (50.7)	169 (49.3)	343
CIN2-3/	136 (87.2)	20 (12.8)	156
CIS/H-SIL/			
SCC	42 (97.7)	1 (2.3)	43
AC	9 (69.2)	4 (30.8)	13
Total	454 (52.2)	416 (47.8)	870

Numbers between brackets represent percentage values.
NIL = no intraepithelial neoplasia;
CIN = cervical intraepithelial neoplasia;
SIL = squamous intraepithelial lesion.
CIS = carcinoma in situ;
SCC = squamous cell carcinoma;
AC = adenocarcinoma.

HPV vaccines, namely HPV 16 and 18, a cocktail of probes for other high risk HPVs and a cocktail of probes for low-risk types. (See Methods for the complete list). As shown in Table 3, we found that HPV 16 was the most prevalent type in invasive cancers as well as in high-grade lesions, accounting for 69.0 % and 58.8 of positive cases, respectively. By contrast, HPV 18 was present in only 4.8% of SCC and 7.3% of high-grade lesions, although, in line with data in the literature, the prevalence of this type was somewhat higher in AC. Thus, these two genotypes, alone or in mixed-type infections, were present in 73.8% of SCC cases and in 66.1% of high-grade lesions. On the other hand, 26.2% of SCC and 35.3% of high-grade lesion were positives for other high-risk HPV types, either alone or in combination with HPV 16 or 18.

As expected, HPV 16 was present in a smaller percentage of low-grade lesions and NIL cases (22.9 and 16.1%, respectively), while, surprisingly, HPV 18 was more frequently found in these (12.6 and 15.0% of CIN1/L-SIL and NIL, respectively), as opposed to high-grade lesions and SCC.

Importantly, HPV types other than 16 and 18 were present in about 2/3 of low-grade lesions.

Table 3: Prevalence of HPV 16 and 18 among HPV positive cervical samples

	HPV 16 [a]	HPV 18 [a]	Other HR [a]	Mixed HR	Mixed HR/LR	LR	ND
NIL N = 93	15 (16.1)	14 (15.0)	56 (60.2)	13 (13.9)	16 (17.2)	33 (35.5)	4 (4.3)
CIN1/L-SIL N = 174	40 (22.9%)	22 (12.6)	81 (46.5 %	23 (13.2)	24 (13.8)	37 (21.2)	10 (5.7)
CIN2-3/CIS/H-SIL/ N = 136	80 (58.8)	10 (7.3)	48 (35.3)	9 (6.6)	1 (0.7)	5 (3.6)	4 (2.9)
SCC N = 42	29 (69.0)	2 (4.8)	11 (26.2)	1 (2.4)	--	--	1 (2.4)
AC N = 9	4 (44.4)	2 (22.2)	3 (33.3)	--	--	--	--

HR = High-Risk HPV types; LR = Low-Risk HPV types; NIL = no intraepithelial neoplasia; CIN = cervical intraepithelial neoplasia; SIL = squamous intraepithelial lesion. CIS = carcinoma in situ; SCC = squamous cell carcinoma; AC = adenocarcinoma. . Numbers between brackets represent percentage values.

[a] As single genotype or as mixed infections.

Table 4: Prevalence of 25 anogenital HPV in cervical samples

HPV type	CIN1/L-SIL	CIN2-3/CIS/H-SIL/	SCC	AC
HPV 6	8 (12.7)	-	-	-
HPV 16	16 (25.4)	41 (56.9)	29 (69.0)	4 (44.4)
HPV 18	7 (11.1)	5 (6.9)	2 (4.8)	2 (22.2)
HPV 30	1 (1.6)	-	-	-
HPV 31	9 (14.3)	4 (5.5)	1 (2.4)	-
HPV 33	1 (1.6)	9 (12.5)	3 (7.1)	-
HPV 35	3 (4.7)	2 (2.8)	1 (2.4)	-
HPV 40	1 (1.6)	-	-	-
HPV 42	15 (23.8)	1 (1.4)	-	-
HPV 43	1 (1.6)	-	-	-
HPV 44	2 (3.2)	-	-	-
HPV 45	1 (1.6)	2 (2.8)	2 (4.8)	1 (11.1)
HPV 51	4 (6.3)	6 (8.3)	1 (2.4)	1 (11.1)
HPV 52	1 (1.6)	1 (1.4)	-	-
HPV 56	6 (9.5)	-	1 (2.4)	-
HPV 58	2 (3.2)	2 (2.8)	-	-
HPV 59	1 (1.6)	-	-	-
HPV 68	-	1 (1.4)	-	-
HPV 70	1 (1.6)	-	-	-
HPV 73	1 (1.6)	1 (1.4)	2 (4.8)	-
ND	3 (4.7)	2 (2.8)	1 (2.4)	-
Total	63	72	42	9

HPV types 11, 26, 34, 39 and 54 were not detected in these samples. Numbers between brackets represent percentage values.; NIL = no intraepithelial neoplasia; CIN = cervical intraepithelial neoplasia; SIL = squamous intraepithelial lesion. CIS = carcinoma in situ; SCC = squamous cell carcinoma; AC = adenocarcinoma; ND = Not determined.

In order to investigate the real prevalence of specific HPV types, in addition to HPV 16 and 18, we fully genotyped a randomly selected group of low-grade (63) and high-grade (72) lesions, as well as all cases of invasive cancer. As shown in table 4, HPV 16 was still the most prevalent type in this selected group of low-grade lesions (25.4%), however the low-risk type HPV 42 was almost equally represented. Importantly, as evident from tables 3 and 4, when compared to other high-risk types, HPV 16 prevalence increases sharply according to the severity of the atypia, going from about 16% in the NIL group, to almost 70%

in SCC cases. This data are in line with the findings of others (20) and suggest that infections sustained by HPV 16 are more prone to persist and cause progressive lesions as compared to other types. Conversely, the prevalence of HPV 18 showed an opposite trend in squamous lesions, being more prevalent in low as opposed to high-grade lesions and SCC.

In fact, in the present study, HPV 18 was found in less that 5% of cases of invasive SCC, similarly to both HPV 45 and 73. In our study, the most prevalent type in both SSC and high-grade lesions, after HPV 16, was the closely related type HPV 33 (7.1% and 12.5%, respectively), while HPV 31, 35, 51 and 56 were each found in only one case (1/42, 2.4%). As shown in table 4, overall, the spectrum of high-risk HPV types was much broader in low-grade lesions and NIL, as compared to High-grade lesions and invasive cancer. As a final point, since the only HPV vaccine currently approved by FDA in addition to HPV 16 and 18, also targets the low-risk types HPV 6 and 11, the genotypes most notoriously associated to the development of condilomata acuminata (genital warts) [24, 25], we wanted to assess the prevalence of these HPV types in tissue biopsies and cell scrapings from lesions of external genitalia and the perianal region from both male and female patients. As shown in Table 5, and in keeping with established data, 80% (52/65) of genital warts analyzed in our study contained the DNA of either HPV 6 or 11, while high risk genotypes or other low-risk types were found in a minority of cases, either alone or associated with HPV 6/11. These data indicate that immunization with an HPV vaccine targeted to genotypes 6 and 11, in addition to 16 and 18, besides representing a valuable tool in the prevention of a great proportion of cervical cancers could be extremely effective in the prophylaxis of genital warts in our population.

Table 5: Prevalence of HPV genotypes in genital warts of external genitalia and the perianal region.

HPV Type	HPV Prevalence
HPV 16	4 (7.0)
HPV 18	-
Other HR	6 (9.2.)
HPV 6/11	52 (80.0)
Other LR	6 (9.2)
Mixed	8 (12.3)
Negative	6 (9.2)
Total	65

HR = High-Risk HPV types; LR = Low-Risk HPV types; Numbers between brackets represent percentage values.

DISCUSSION

Within the last 2 decades, progress in the understanding of cervical cancer biology has had a profound effect on how we approach this disease. The notion that infection with high-risk HPV types is a necessary, though not sufficient, step in the development of cervical as well other cancers of the anogenital tract has already forced us to reconsider screening strategies for these diseases. More recently, the arrival on the market of anti-HPV prophylactic vaccines promises to revolutionize current preventive strategies for HPV-related cancers. Unfortunately, not unlike other highly adapted human viruses, HPV exists in nature in a great number of serotypes and variants that elicit largely non-cross-reactive, type-restricted immune responses [12]. Thus one of the major challenges to the success of HPV vaccines would be their range of type-effectiveness.

Current HPV prophylactic vaccines were designed so as to target only two high-risk HPV types, with the notion that these represent the most prevalent types in cervical cancer cases worldwide [7, 10]. However differences in the prevalence among different populations are well known and are likely to have an impact on vaccine efficacy. Thus, actual prevalence data in different populations can be very valuable in monitoring vaccine efficacy during clinical studies and they could also be instrumental in designing more effective, population-tailored vaccine formulations that incorporate the most prevailing types in any given geographical area.

The main goal of our work was to gather information on the prevalence of HPV 16 and 18, the two high risk HPV types included in the prophylactic vaccines under clinical trial, in those cervical lesions that are currently considered for ablative treatment, namely CIN2/HSIL or higher, in order to be able to predict the possible clinical benefits brought by the implementation of the HPV vaccine in our region. To the best of our knowledge this is the largest such study ever reported on Italian women, including over 200 cases of high-grade cervical lesions and invasive carcinomas. In keeping with published reports in Italian women and worldwide [20, 26], the prevalence of HPV infection in our study varied according to the degree of cervical atypia, approaching 100% in squamous cell carcinoma, although, as reported in most studies [10], prevalence remained significantly lower in ACs.

Almost 30% of women with NIL smears were positive for HPV, a value somewhat higher than

expected for the general population of this age group (mean age 37.1 years). However, though negative for intraepithelial lesion or malignancy, the women of this group underwent reflex HPV DNA testing because of non-specific alterations in their smears. As such they represent a selected group of women that cannot be considered representative of the general population.

In line with known epidemiological data from Italy [26-28] and other western Countries [7, 10], we found that HPV 16 is by far the most prevalent type in SCC and high-grade lesions, accounting for almost 70% of positives cases among SCCs in our study. Conversely, in contrast to most epidemiological surveys [7,10], our data would suggest that HPV 18 does not play a major role in the development SCC in our population. In fact, in our study the prevalence of this HPV type was much higher in low-grade lesions as compared to high-grade squamous lesions and invasive carcinomas. It is obvious that the natural history of HPV 18 infection in our population cannot be inferred solely by single point prevalence data, however our findings would suggest that, in contrast to what reported by others [29, 30], infection with this HPV type is not associated with highly progressive lesions in our population. It is obvious that the prevalence of some high-risk HPV types might be underestimated in high-grade lesions, as compared to low-grade lesions and NIL, due to the small number of cases examined. Nonetheless, their relative rarity in these lesions may reflect their scarce tendency to cause persistent infections and progressive pre-invasive lesions.

In summary, our data indicate that almost 75% of SCC and 65% of high-grade lesions in our region are due to infection with either HPV 16 or 18. Assuming that immunization programs will achieve 100% protection rates against infection with either HPV 16 or 18 in vaccinated women (as reported in phase III clinical trials), at least in theory we could expect to prevent up to 75% of cervical cancers solely by vaccination, in our region. Obviously, these theoretical considerations will have to be weighted against the degree of vaccine coverage actually reached in the population at risk. The true challenge for the near future will be to obtain the proper extent of vaccine coverage in those women who are also less likely to adhere to screening programs and as such are at greater risk of cervical cancer.

After HPV 16, the viral type most frequently found in SCCs in our study was HPV 33, a type not included in current vaccine formulations, but closely related to HPV 16. Interestingly, recent reports on phase III clinical trials [17] would suggest that the bivalent GSK vaccine might be able to offer limited but significant protection against HPV types closely related to either

HPV 16 (such as HPV 31) or HPV 18 (such as HPV 45). While further studies are needed to confirm these reports, our data indicate that about 1/4 of SCC and 1/3 of high-grade lesions harbor HPV types not included in current vaccine formulation, thus, even in vaccinated populations, screening for cytological alteration or/and HPV DNA will continue to be a very important tool in the fight against cervical cancer. In fact, recently issued American Cancer Society guidelines [31] recommend that "women undergo regular screening regardless of whether they have been vaccinated".

Screening programs based on Pap smears and HPV DNA tests will continue to be the main preventive tool for women 19 or older, in which vaccination is not recommended [31]. In this contest, it is important to note that our data show a clear trend towards increasing prevalence values of HPV 16 infection with increasing grades of atypia. We take this as indirect evidence that HPV 16 is much more prone to cause persistent infections and progressive lesions, as compared to other high-risk types, a conclusion well supported by more direct evidence from prospective studies [32-34]. Since viral persistence is considered necessary prerequisite for the development of progressive lesions [35] and since it is estimated that up to 30% of women infected with persistent HPV 16 will develop high-grade lesions or cancer within 10 years [32, 33], we, as others [34], suggest that the women persistently infected with HPV 16 may deserve a closer follow-up as compared to those infected with other high-risk types, even in the absence of any Pap smear alteration.

Genital warts, although entirely benign, represent a major factor of morbidity and among the most common sexually transmitted diseases. As confirmed in this paper, the vast majority of these lesions are caused by infection with HPV 6 or 11 [24, 25]. In this respect, the association of these two HPV types with HPV 16 and 18 in a polyvalent vaccine formulation may prove to be a clever strategy for the prevention of this disease in addition to cervical cancer.

In conclusion, although many important issues, such as cost/benefit analysis, vaccine delivery and acceptance by the target population (and the communities), still await to be fully investigated, from a clinical standpoint, the introduction of HPV vaccines is likely to offer a very effective tool for the prevention of cervical as well as other HPV related neoplasias. Whether this will translate in net gains for the women and the health care system in our geographical area, will depend largely on the choice of the right vaccine implementation strategies.

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