



## Distribution of human papillomavirus genotypes in invasive cervical cancer in Italy: A representative, single institution case series

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### ABSTRACT

Despite worldwide human papillomavirus (HPV) types distribution showed constant rates of HPV 16/18 in cervical cancers, regional variations have been consistently documented. Very little data is available on HPV genotype prevalence among Italian women with invasive cervical cancer. This study aims to determine the HPV type distribution in cervical specimens obtained from Italian women diagnosed with invasive cervical cancer and referred to the European Institute of Oncology (IEO).

Two hundred-sixty eight cervical specimens were obtained from patients diagnosed with invasive cervical cancer referred to the European Institute of Oncology between 1996 and 2006.

Following preparation, all cervical samples were sent to laboratories at the International Agency for Research on Cancer (IARC, Lyon, France) for DNA extraction and HPV typing by the multiplex PCR/APEX assay. The study population was divided into four groups from different macro regions: (i) Milan and surrounding area ( $n = 57$ , 21.3%), (ii) northern Italy ( $n = 81$ , 30.2%), (iii) central Italy ( $n = 64$ , 23.9%) and (iv) southern Italy ( $n = 66$ , 24.6%).

The present study is the first at our knowledge that examines a fair number of Italian cervical cancers, about one tenth of all estimated cervical cancer cases occurring yearly, distributed across the whole country. Two-hundred and fifty-one patients (93.7%) resulted HPV DNA positive; of these 201 patients (80.1%) presented a single infection, whereas 50 women (19.9%) presented multiple infection. One hundred and eighty-nine specimens (75.3%) tested positive for either HPV 16 or HPV 18, whereas 62 (24.7%) resulted positive for other high-risk HPV genotypes only. The proportion of HPV 16/18 positive invasive cervical cancers was similar for all the four geographical Italian areas considered. A statistically significant association with younger age and earlier stage was observed for HPV 16/18 related invasive cervical cancers.

The results demonstrate that the proportion of HPV 16/18 cervical cancers is fairly constant in all the areas and covers more than 70% of Italian cervical cancer cases. This observation strengthens the decision to start the vaccination programme in all the Italian regions. In addition, the present study provides new and original data on the genotype related differences of the disease that are worth of further investigation.

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### 1. Introduction

Cervical cancer affects almost 500,000 women every year, representing the second most common malignancy in the female population worldwide [1,2].

Virtually all cervical cancer cases result from persistent genital infection with human papillomavirus (HPV). There is international consensus that a minority of genotypes of HPV, named “high-risk

types”, are responsible of the vast majority of cases. A survey of 11 case/control studies in 9 countries classified “high-risk types” 15 different HPVs, namely 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 [3]. An additional three HPV types were classified as probable high-risk types, HPV 26, 53 and 66, while several others were considered low-risk types, including HPV 70 that is phylogenetically related to the high-risk HPV types. While HPV 16 and 18 are clearly the two most frequent types detected in cervical cancer worldwide, covering approximately 70% of the cases, the prevalence of the other high-risk HPV types in cervical cancer can vary in different geographical regions. For instance, HPV 45 is the third most frequent genotype in Africa, while the same position is occupied by HPV 58 in Asia and by HPV 33 in Europe [4].

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Current HPV vaccines are designed to protect against the two most common high-risk HPV types, HPV 16 and 18 [5,6]. Prophylactic vaccines induce a HPV-type specific protection [7–10], though a cross-protection of available vaccines has been hypothesized and demonstrated in clinical trials [9–11]. In order to induce the protection against additional high-risk HPV types, VLPs containing L1 from other HPV types should be included in the vaccines. In this scenario, it is outmostly important to determine the precise distribution of the high-risk HPV types in cervical cancer in different countries.

Very little data are available about HPV genotypes prevalence among women with cervical cancer in Italy [12,13]. Aim of the present study was therefore the HPV genotypization of cervical specimens obtained from 268 women diagnosed with invasive cervical cancer, living in Italy and referred for treatment to our Institution. Findings have been stratified according to age, histologic type, stage at diagnosis, geographical area of residence, analysed and discussed.

## 2. Methods

### 2.1. Study population

The study population was represented by 268 patients diagnosed with invasive cervical cancer that have been selected among the patients referred to the European Institute of Oncology (IEO) between 1996 and 2006. Aim of the selection process was to obtain a sample evenly distributed according to age at diagnosis and macro-area of residence. Written informed consent for the use of tissue samples for study and research is routinely obtained at IEO at enrolment and it has been obtained for all women included in the study.

### 2.2. Preparation of the paraffin samples

After the sampling, the cervical specimens were embedded in paraffin. Five sections 5  $\mu$ m thick have been obtained from each paraffin block. To assure that HPV typing is performed onto DNA extracted from cancer tissue, the first and the last section of the series have been stained and read by the pathologist, while the internal sections have been processed for DNA extraction. To reduce the risk of cross-contamination, the blades have been frequently changed and the microtome extensively washed with water/ethanol solutions. To detect possible cross-contaminations empty paraffin blocks have been cut every five cancer specimens and carefully analysed for HPV and  $\beta$ -globin positivity [14].

### 2.3. DNA extraction and HPV typing

Following preparation all cervical samples have been labelled and sent to laboratories at the International Agency for Research on Cancer (IARC, Lyon, France) for HPV typing. DNA was prepared by incubating the paraffin tissue sections in digestion buffer (10 mM Tris/HCl pH 7.4, proteinase K 0.5 mg/ml and Tween 20 0.4%) overnight at 37 °C.  $\beta$ -Globin PCR analysis has been performed to

evaluate the quality of DNA preparation as described by Gheit et al. [14]. HPV typing used the multiplex PCR/APEX assay. Briefly, multiplex PCR has been performed using the Qiagen Multiplex PCR Kit according to the instructions of the manufacturer, with a mixture containing 19 pairs of HPV-type specific primers (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82) following the procedure described by Gheit et al. [14]. To increase the sensitivity of HPV 16 detection we modified the PCR forward primer for HPV 16 E7 (5'-TTATGAGCAATTAATGACAGCTCAG-3') as described in Cazzaniga et al. [15]. The modified version of the multiplex PCR assay showed a comparable sensitivity to the GP5+/6+ reverse line blot assay in detecting HPV 16 DNA (data not shown).

### 2.4. Statistical analysis

Pearson's chi square test was used to compare the HPV genotypes found in the cervical cancer cases divided by International Federation of Gynecology and Obstetrics (FIGO) stage (I stage vs. stages >I) and by age of the patient ( $\leq 35$  years vs. >35 years old).

## 3. Results

A total of 268 specimens were analysed. The age range of the women included in the study was 23–76 years, with a median age of 46 years.

According to the study design women were divided in different sub-groups according their geographical locations. Four groups coming from the following macro-areas were identified: (i) Milan and surrounding area ( $n = 57$ , 21.3%), (ii) northern Italy excluding Milan area ( $n = 81$ , 30.2%), (iii) central Italy ( $n = 64$ , 23.9%) and (iv) southern Italy, including Sardinia and Sicily islands ( $n = 66$ , 24.6%). Two hundred and fifty one patients (93.7%) were found positive for the HPV DNA with the multiplex PCR/APEX assay. Table 1 summarizes the distribution and percentage of HPV DNA positive cases and the distribution of HPV 16 and 18 as compared to other HPV genotypes among the different geographical Italian macro-areas considered.

The most represented histotype was squamous cell carcinoma (257 cases), of these 241 (93.8%) resulted HPV DNA positive. HPV 16 and/or 18 was found in 75.1% (181 cases) of HPV DNA positive squamous cell carcinomas, while other HPV genotypes only were found in 60 cases (24.9%). The other histotypes represented were two adenocarcinomas, both HPV 18 positive, five adenosquamous carcinoma (two positive for HPV 18, two positive for HPV 16, one positive for HPV 45) and other minor histotypes (four cases), with one HPV DNA negative specimen.

Stage of disease at diagnosis according the FIGO classification was mostly stage I, with 33 specimens at stage IA (12.3%) and 173 at stage IB (64.6%). The cervical carcinomas at more advanced stages were 61 (22.8%), with 50 specimens at stage II (18.7%) and 11 cases at stage III or IV (4.1%). We had no information of the FIGO stage at the diagnosis of one of the analysed carcinomas; this case resulted HPV negative. Of the 17 HPV DNA negative specimens 9 were at

**Table 1**

Number and percentage (%) of HPV positive and distribution of HPV 16 and/or 18 as compared to other HPV genotypes in the studied population (HPV DNA+), among the different Italian macro-areas of origin.

Area	Number of HPV DNA+ (%)	Percentage of patients HPV DNA+ (CI 95%)	HPV 16-18 (%)	Other genotypes (%)
North	74 (29.5)	91.36 (83.00–96.45)	52 (27.5)	22 (35.4)
Central	62 (24.7)	96.88 (89.16–99.62)	49 (25.9)	13 (21.0)
South and major Islands	61 (24.3)	92.42 (83.20–97.49)	45 (23.8)	16 (25.0)
Milan and surrounding	54 (21.5)	94.74 (85.38–98.90)	43 (22.7)	11 (17.7)
TOT	251 (100)	93.65 (90.73–96.56)	189 (100)	62 (100)

**Table 2**  
Distribution of HPV genotypes among the population positive for HPV DNA.

Genotype	Single infections	Multiple infections
16	131	39
18	15	15
31	13	20
33	5	5
35	3	3
39	2	1
45	10	6
51	–	4
52	3	2
53	–	2
56	2	1
58	3	3
59	4	–
66	1	2
68	2	1
70	2	–
73	5	3
82	–	1

stage IB (52.9%), 5 at stage II (29.4%) and 2 were more advanced (11.8%).

Tables 2 and 3 summarize the distribution of HPV genotypes among the HPV DNA positive population considering single and multiple infections. Two-hundred and one patients (80.1%) presented a single infection, whereas 50 specimens (19.9% of the cases) presented multiple HPV genotypes infection.

Considering only the positive samples, 189 specimens (75.3%) were positive for either HPV 16 or HPV 18, whereas 62 (24.7%) resulted positive for other HPV genotypes only.

Table 4 shows the distribution of HPV 16–18 compared to other HPV types considering the age at the diagnosis of the studied patients. HPV 16 and/or 18 were significantly more frequent at 35 years old or less than at older ages, while the other HPV genotypes were more present after 35 years old than at younger ages. It is of interest to note that 87.2% of invasive cancers in the women  $\leq 35$  years old were 16 or 18 related. This difference was statistically significant (Pearson's chi square test:  $p = 0.035$ ). Table 5 describes the studied specimens presenting HPV 16 and/or 18 infections according to the FIGO stage at diagnosis compared to cases positive for other HPV genotypes only. Comparing 251 HPV positive carcinomas

**Table 3**  
Distribution of HPV genotypes among the population positive for a multiple HPV DNA.

Others genotype	Most important genotype present						
	16	18	31	33	35	51	58
18	11 <sup>a,b</sup>	–	–	–	–	–	–
31	14 <sup>c,d,e</sup>	1	–	–	–	–	–
33	3	–	1	–	–	–	–
35	1	–	–	–	–	–	–
39	1	–	–	–	–	–	–
45	3 <sup>f</sup>	–	1	–	1	–	–
51	1	–	–	–	–	–	–
52	1	1	–	–	–	–	–
53	–	–	–	1	–	–	–
56	–	–	1	–	–	–	–
58	2	–	–	–	–	–	–
66	–	1	–	–	–	–	–
68	–	–	–	–	–	1	–
73	2	1	–	–	–	–	–
82	–	–	–	–	–	–	1

<sup>a</sup> Multiple infection genotypes 16–18–31.

<sup>b</sup> Multiple infection genotypes 16–18–31–51.

<sup>c</sup> Multiple infection genotypes 16–31–45.

<sup>d</sup> Multiple infection genotype 16–31–51.

<sup>e</sup> Multiple infection genotypes 16–31–35–53.

<sup>f</sup> Multiple infection genotype 16–45–66.

**Table 4**  
Distribution of HPV 16–18 compared to other HPV types considering the age at the diagnosis of the HPV DNA+ patients.

Genotype	Age		Total (%)
	$\leq 35$ years (%)	$> 35$ years (%)	
16–18	41 (87.2)	148 (72.5)	189 (75.3)
Others	6 (12.8)	56 (27.5)	62 (24.7)
Total (%)	47 (100)	204 (100)	251 (100)

Pearson's chi square test  $p = 0.035$ .

**Table 5**  
FIGO stage of 251 HPV positive patients, according to the HPV genotype.

FIGO stage	HPV 16–18 (%)	Other HPV types (%)
IA	27 (14.3)	6 (9.7)
IB	128 (67.7)	36 (58.1)
II	29 (15.3)	16 (25.8)
III–IV	5 (2.7)	4 (6.4)
Total	189 (100)	62 (100)

samples at different stages, HPV 16–18 were found more frequently at stage I than at more advanced stages; while HPV non-16 or 18 were more frequent at advanced stages. According to Pearson's chi square test this difference was statistically significant ( $p = 0.018$ ).

HPV 31 and 45 represent an important subset of infection; the prevalence of these two genotypes varies quite extensively among the different geographical regions worldwide. If we exclude the multiple infections comprehending HPV 16 and/or 18, in our series there were 24 cases who tested positive for either HPV 31 (13 patients), HPV 45 (10 patients) or HPV 31 and 45 (multiple infection, one case); of these HPV 31 and/or 45 positive specimens 22 were squamous cell carcinomas, whereas one was an adenosquamous carcinoma and another one a so called lymphoepithelial carcinoma of the cervix. One of these cases was at FIGO stage IA (4.2%), 16 at stage IB (66.7%), 5 at stage II (20.8%) and 2 at stage III or IV (8.3%).

There were eight specimens testing positive for HPV 33, 52 or 58, excluding the multiple infections. All of the patients infected by those genotypes presented with squamous cell carcinomas and only two had a FIGO stage IIB at diagnosis the other being mostly stage IA and IB. There was no substantial difference among the geographical distribution of HPV 33, 52 and 58, even taking into account the small numbers involved.

#### 4. Discussion

Despite HPV types distribution in tumours worldwide showed constant rates of HPV 16/18 in cervical cancers, 70.7% in all regions combined, regional variations have been consistently observed. The proportion of cases attributed to these two types are 63.9% in sub-Saharan Africa, 78.9% in northern Africa, 65.0% in central/south America, 73.5% in south Asia, 71.5% in Europe/north America [4]. Italy is very heterogeneous extending from the continental north to insular areas and a southern part located in the middle of the Mediterranean Sea. Therefore the possibility of a regional variation in HPV type distribution in cervical cancers in Italy cannot be disregarded. With the recent initiation of the vaccination programme in Italy, knowing the distribution of HPV type in Italian cervical cancers is therefore important. The estimated number of cervical cancer occurring in Italy yearly is about 3500. The present study is the first at our knowledge that examines a fair number of Italian cervical cancers, about one tenth of all cancer occurring yearly, in all the four Italian macro-areas evaluated. The results of the present study are based on a series of cervical cancer samples, collected in a single institution and, more important, HPV DNA detection was done in a

single laboratory using a PCR-based assay. This is of great advantage because it strengthens the data obtained; furthermore the present analysis has been conducted at the IARC and therefore the results are easily comparable with similar series from other countries or other already published data. The results strongly indicate that the proportion of HPV 16/18 cervical cancers is fairly constant in all the areas and covers 70.52% of cervical cancer cases. This observation strengthens the decision to start the vaccination programme in all the Italian regions, since it confirms the projected impact on cancer prevention expected by 11 years old girls vaccination programme. This series of cervical cancer enabled us to investigate HPV type distribution in relation to some clinical features. An unexpected significant result was the finding that HPV 16/18 cervical cancers occur more frequently at younger ages. This result is in keeping with the observation that HPV 16 related CIN3 is observed at young ages, relatively early after HPV acquisition [16]. A second unexpected significant result was the finding that non-HPV 16/18 cervical cancers were more frequently observed as advanced stage. We do not know if this result is an occasional finding or it could be replicated in other series. Moreover, it could be influenced by age but due to the limited number of cases, specially those with an advanced FIGO stage in the group of 35 years old patients or younger, we could not stratify for age. Indeed, there is some evidence that HPV 16 related precancerous lesions are more easily diagnosed than CIN not related to HPV 16 [17,18]. In this way HPV 16 related cervical cancers may be better and earlier identified by cytological screening and therefore diagnosed at an earlier stage than non-HPV 16 related cervical cancers.

In conclusion the present investigation confirms also for the Italian population the data collected from other European countries on the genotype distribution in invasive cervical cancers. In addition, the present study shed new light on the genotype related differences of the disease that are worth of further investigation.

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