Type-Specific Human Papillomavirus Distribution in Invasive Squamous Cervical Carcinomas in Tunisia and Vaccine Impact

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RESEARCH ARTICLE

Type-Specific Human Papillomavirus Distribution in Invasive Squamous Cervical Carcinomas in Tunisia and Vaccine Impact

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Abstract

Background: High risk human papillomaviruses (HPVs) are the leading cause of cervical cancer (CC) and Pap smear screening has not been successful in preventing CC in Tunisia. HPV vaccination that targets HPV16 and 18 offers a new efficient prevention tool. Identification of HPV types in CC is thus essential to determine the impact of HPV vaccine implementation. The aim of this study is to provide specific data from Tunisia. Materials and Methods: A total of 89 histological confirmed paraffin embedded samples isolated from patients with CC diagnosed between 2001 and 2011 were collected from five medical centres from Northern and Southern Tunisia. HPV DNA was detected using a nested PCR (MY09/MY11-GP5+/GP6+) and genotyping was assessed using a reverse blot line hybridisation assay that enables the detection of 32 HPV types. Results: HPV DNA was detected in all samples. Twelve high risk types were detected; HPV16 and/or 18 were predominant, accounting together for 92.1% of all the CC cases (HPV16: 83.1%). Single infections accounted for 48.8% of the cases and were mostly linked to HPV 16 (32.6%) and less frequently to HPV 18 (2.4%). The other high risk HPV single infections were linked to HPV 35 (4.6%), 45 (4.6%), 58 (2.3%) and 59 (2.3%). Multiple infections with mixing of 2 to 4 genotypes predominately featrued HPV16 and/or 18 with HPV 35 and 45 (96.6 %) and less frequently with HPV 59, 40, 66, 73 and 58. There was no statistically significant variation in the relative distribution of HPV types with age. Conclusions: These results strongly indicate that prophylactic HPV vaccines can have a major impact in preventing CC in Tunisia

Keywords: Cervical cancer - human papillomavirus - genotypes - vaccine - Tunisia

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Introduction

HPV infection is the most common sexually transmitted infection all over the world and persistent infection with high risk HPV (HR-HPV) is the cause of cervical cancer (CC) (Walboomers M et al., 1999; Cogliano et al., 2005). The estimates CC burden in the world indicate an incidence of 529.000 cases/year and 275.000 deaths/year (Ferlay et al., 2010). Eighty percent of CC cases are killing women in under-resourced countries. Future projections have statute that the incidence will reach 1.000.000 cases/year in 2050 (Feraly et al., 2010). Among Tunisian women, this cancer now ranks third after breast and colorectal cancer (Ben Abdalah et al., 2009). However, since preventable, it is considered as a public health priority. In Tunisia, Pap smear screening programs have not been successful in preventing CC so the introduction of prophylactic HPV vaccination needs to be considered. Prophylactic HPV vaccines are designed to prevent HPV 16 and 18 infections and are considered efficient in reducing the burden of CC. Despite a constant rate distribution of HPV 16 and 18 in CC all over the world (Kulkarni et al.,2011; Shen et Liu., 2013; Abdul Raub et al.,2014), regional variations have been documented (Clifford et al., 2003; Clifford et al., 2006). Thus, identification of HPV genotypes in CC is of great importance to determine the effectiveness of prophylactic vaccines implementation. The aim of the present study is therefore to determine using standardized protocols, HPV genotypes distribution in Tunisian specimens of squamous cell carcinoma (SCC) the most represented histotype of CC.

Materials and Methods

Study population

The study was conducted at Pasteur Institute of Tunis (IPT), in the HPV unit Research of the "Laboratory of Molecular Epidemiology and Experimental Pathology applied to infectious diseases" with straight collaboration of the Department of Human and Experimental Pathology.

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Recruitment was performed in the period ranging from 2001 to 2011. 89 archival selected paraffin embedded samples of diagnosed SCC were collected. The aim of the selection process was to avoid Bouin fixation that is unfortunately widely used and that causes DNA alterations (Longy et al., 1997; Benerini Gatta et al 2012). Biopsies and surgical specimens were assembled from 4 medical centers across the country: 68 samples from 3 Northern centers (24 from IPT, 30 from La Marsa hospital and 14 from Nabeul hospital) and 21 samples from the hospital of Sfax located in the center of Tunisia. The diagnosis of primary SCC was confirmed by histopathology for all samples.

Preparation of the paraffin samples

To ensure that HPV typing is performed onto DNA extracted from cancer tissue, a 4 μm thick section was stained with haematoxylin and eosin and evaluated by a pathologist of the Department of Human and Experimental Pathology (IPT). Four other sections of 10 µm thick each were further obtained from each paraffin block and kept in Eppendorf tubes for DNA extraction. To reduce the risk of cross-contamination, the blades were changed for each specimen and the microtome extensively washed with ethanol solutions. For each sample, a deparaffinization process eliminates the surrounding paraffin edges of the tissue section using a sterile crowbar. Then 3 washing steps in 65° Xylène and 4 washing steps with decreasing concentrations of ethanol were achieved at room temperature. A final heating of the samples was performed at 45°C to exclude paraffin remnants.

DNA extraction, HPV DNA detection and typing

DNA extraction was performed using the QIA amp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA quantification was assessed by spectrophotometry (A260/A280). HPV detection was performed with a nested PCR using MY09/MY11 and GP5+/GP6+ biotinylated primers as described by Carvalho Nde O et al (Carvalho Nde et al., 2010) The first PCR was performed in a final reaction volume of 50 µL, containing 10 to 25 ng of DNA, 10 µL of buffer [100 mM Tris-HCl, 500 mM KCl], 50 μM of $MgCl_2$, 2.5 μM of dNTPs, 10 μM of each primer and 2.5 U of Taq DNA polymerase. The second PCR was performed in a final volume of 50 µL, containing 1 µL of the first reaction, 10 µL of buffer [100 mM Tris-HCl, 500 mM KCl], 50 μM of $MgCl_2$, 2.5 μM of dNTPs, 10 μM of each primer and 2.5 U of Taq DNA polymerase.

Genotyping was performed by a Reverse Line Blot hybridization assay using the different biotinylated probes in a Miniblotter system (World Health organization, 2009; Eklund et al, 2010). The oligonucleotides were covalently joined to a Biodyne C membrane and activated with EDAC reagent (Sigma). The PCR products were denatured at 96°C, cooled on ice, and applied to each capillary of the Miniblotter. Hybridization was completed after 1 hour and the signal (peroxidase conjugate streptadivin Roche) was detected using a chemiluminescent autoradiography. This hybridization assay was performed for HLADQ and 32 HPV genotypes: HPV 6 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54; 55, 56, 57, 58, 59a, 59b, 66, 68, 69, 70, 73, 82, 83, 84.

Statistical analysis

All statistical analyses were performed using SPSS 13.0. Descriptive analysis was performed and the proportion of different genotypes was calculated. Comparison of mean age according to HPV genotypes was achieved using T-test. Chi-square test was used to compare the distribution of different genotypes by age groups.

Results

HPV detection

The study was conducted among 89 women aged from 33 to 88 years (mean 56.47 ± 12.86 years). HPV DNA was detected in all samples using nested PCR. Thus, among all subjects of this study, the prevalence of HPV infection in SCC was 100 %. Sixteen HPV genotypes were detected including 12 high risk types. The most prevalent genotypes were HPV16 and HPV 18 detected in respectively 83.1% and 42.7% of samples. When both single and mixed infections were considered, HPV16 and/or HPV18 were detected in 92.1% of the SCC. Single infections accounted for 48.8% of the specimens and were mostly linked to HPV 16 (32.6% of single infections) and less frequently to HPV 18 (2.4%). The others HR-HPV single infections

Table 1. Proportional Distribution of HR-HPV Types that were Detected in this Series of Invasive Cervical Carcinoma

			Single infections		Mixed infections	
HR-HPV type	Total	%	number	%	number	%
16	66	86.6	26	34.2	40	52.6
18	35	46	6	7.89	29	38.1
35	20	26.3	21	2.63	18	23.6
45	17	22.3	0	1.3	16	21
59	14	18.42	0	0	14	18.4
66	8	10.52	0	0	8	10.52
73	6	8.45	0	0	6	8.45
58	7	9.2	0	0	7	9.2
53	5	6.5	0	0	5	6.5
68	4	5.2	0	0	4	5.2
33	1	1	0	0	1	1
31	1	1	0	0	1	1

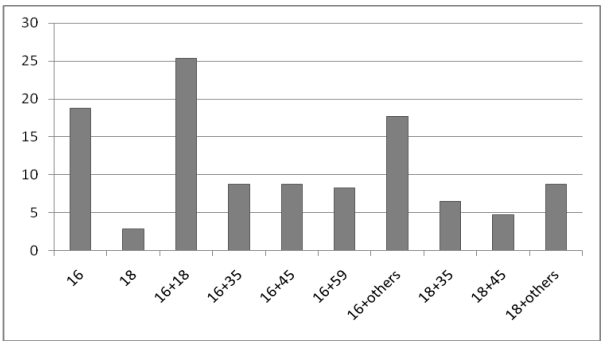

Figure 1. Proportional Distribution of Single and Mixed Infections with HPV16 and HPV 18 in this Series of Squamous Invasive Cervical Carcinoma

were linked to HPV 35 (4.6%), 45 (4.6%), 58 (2.3%) and 59 (2.3%). Multiple infections were mixing 2 to 4 HPV types and were predominately mixing HPV16 and/or 18 with HPV 35 and 45 (96.6 %), and less frequently HPV16 and/or 18 with HPV 59, 40, 66, 73 and 58 (Figure 1). Four low risk HPV types (HPV44, 40, 6 and 11) were detected in 22.4 % of SCC and were always mixed with HPV 16 and/or HPV18 and/or HPV35. The distribution of HR-HPV types is reported in table 1.

Statistical results

There was not any significant change in the relative distribution of the detected HPV types according to age using Chi-square test.

Discussion

The Tunisian CC incidence is similar to that seen in developed countries despite much lower screening (Cancer incidence in Five Continents, vol IX, 2007).According to the 2008 North cancer Tunisian registry of cancer, CC has moved from second to third women cancer after breast and colorectal cancer (Ben Abdallah et al., 2009). However, there is no statistics indicating a real decrease of its incidence and CC remains a national health problem and a national priority as it is a preventable cancer. Pap smear screening programs have not been efficient in preventing the disease (Njah et al, 1994; Hsairi et al., 2013; Lazzar et al., 2014) and vaccine implementation, considered as a valuable tool for CC control (Human Papillomavirus vaccines. WHO, 2009) is under investigation in Tunisia. Thus, collecting epidemiological data and information about HPV genotype distribution in CC is the keys to study the effectiveness of prophylactic strategies such HPV vaccination. HPV16 and 18 are the target of the two affordable HPV prophylactic vaccines and they have been established as the most common HPV types found in CC worldwide despite consistent regional variations (Munoz et al., 2004; De Sanjose et al., 2010; Sjoeborg., 2010). Efficacy of these vaccines in reducing CIN2/ CIN3 lesions associated with HPV 16 and 18 types has been estimated to 93%-100% (Harper et al., 2006; Villa et al., 2006).

The first North African report describing HPV genotypes in CC was from Morocco (Chaouki et al., 1998). The study published in 1998, shows that HPV 16 and 18 are predominant (53.3% and 7.9 %). The second (Hammouda et al., 2005) reports a majority (95 %) of HPV 16 and 18 isolates from Algerian patients with CC. In our Tunisian series, we show that HPV 16 and/or 18 are present in 92.13 % of tested SCC. Two previous Tunisian studies showed similar findings. Missaoui et al report (Missaoui et al., 2010) shows using PCR technique and consensus GP5/GP6 primers, that HPV 16 is the most frequent detected genotype (47.6%) in the samples isolated from their patients. HPV 16 and 18 were also the most reported genotypes in a series of 146 cases collected in the Tunisian cancer Institute (KrennHrubec et al., 2011). Our results also show that multiple infections were seen in 51.7 % of cases and were mainly mixing HPV 16, 18, 35 and 45 and less frequently HPV 59, 40, 66, 73 and 58. Multiple infections have to be taken in consideration since the emergence of HPV genotype other than HPV16 and HPV18 after vaccination could alter the effectiveness of vaccination. However, we could also consider that the immune response to HPV vaccine type can lead to a cross-protection against the closely related species. Indeed, HPV 16 belongs to the A9 species which also includes types 31, 33, 52, 58, and 35. HPV 18 belongs to the A7 species which also includes types 45, 59 and 39 and it has recently shown a cross protection for HPV35 and 45 (Paavonen et al., 2009).

At a technical level, while the usual HPV detection rate among formalin fixed and paraffin embedded samples is under 99 %, all the samples tested in our study were positive for HPV DNA. DNA fragmentation in formalin fixed paraffin embedded tissues are significantly more frequent compared to fresh tissues so DNA integrity usually decreases overtime in those samples and the probability to get false negatives increases (Mc Sherry et al., 2007; Fattorini et al., 2009; Webersinke et al., 2013). This can be overcome in part by the use of the general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequence (De Roda et al., 1995; Lee, 2012) or the use of a nested PCR performed with MY09/MY11 and GP5+/GP6+ biotinylated primers as we did in our study (Carvalho et al., 2010). High levels of DNA concentration combined with these PCR conditions allow a detection of HPV DNA in 100 % of the samples. That was close to the findings of the Moroccan study that has been using a similar PCR protocol (Chaouki et al., 1998).

Our study shows that the prevalence of HPV 16 and/or 18 is highly predominant in our Tunisian SCC series suggesting that the current prophylactic vaccines might be potentially effective in preventing the disease. If current screening policies need to be revised in Tunisia, these results indicate that HPV vaccination can have a major impact on CC preventive guidelines. Mixed infections are crucial when considering second generation vaccines with broader coverage of HPV types and the potential benefits of cross-protection with current vaccines. The prevalence of HPV 35 and 45 in our study is interesting to notice when considering the cross protection that has been described with HPV 35 and 45 (Paavonen et al., 2009).

Cost-effectiveness analyses has been done in various parts in the world and has been conducted based on available data of HPV prevalence. The most important surveys have been assembled in a global meta-analysis. Besides differences in the PCR technologies used (Clifford et al., 2006), this analysis has provided relevant information for designing HPV vaccines and determining their impact in all world regions. Further documentation about HPV prevalence at a national level and its distribution according to age is needed to study the cost-effectiveness of vaccine implementation in preadolescent girls and for the initial catch-up efforts in elder women (C Kitchener et al., 2014).

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References

Abdul Raub SH, Isa NM, Zailani HA, et al (2014) Distribution of HPV Genotypes in Cervical Cancer in Multi-ethnic Malaysia. *Asian Pac J Cancer Prev*, **12**, 645-8.

Ben Abdallah M, Zehani S, Hizem Ben Ayoub W (2009). Registre des Cancers Nord-Tunisie.

Benerini Gatta L, Cadei M, Balzarini P, et al (2012) Application of alternative fixatives to formalin in diagnostic pathology. *Eur J Histochem*, **56**, 12.

Cancer incidence in Five Continents, vol. IX. (2007). in "the Narratives and the Maps, Africa" Eds Curado.M.P, Edwards, B, Shin H.R., Storm H et al. WHO Press. Switzerland pp 107 Numbers 160 IARC Scientific Publications

Carvalho Nde O, del Castillo DM, Perone C, et al (2010). Comparison of HPV genotyping by type-specific PCR and sequencing. *Mem Inst Oswaldo Cruz*, **105**, 73-8.

C Kitchener H, Canfell K, Gilham C, et al (2014). The clinical effectiveness and cost-effectiveness of primary human papillomavirus cervical screening in England: extended follow-up of the ARTISTIC randomised trial cohort through three screening rounds. *Health Technol Assess*, **18**, 1-196.

Chaouki N, Bosch FX, Muñoz N, et al (1998). The viral origin of cervical cancer in Rabat, Morocco. *Int J Cancer*, **75**, 546-54.

Clifford GM, Smith JS, Plummer M, Munoz N, Francheschi S (2003). Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *British Journal of Cancer*, **88**, 63-73

Clifford G, Franceschi S, Diaz M, Munoz N, Villa LL (2006) Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. *Vaccine*, **31**, 26-34.

Cogliano V, Baan R, Straif K, et al (2005). WHO International Agency for Research on Cancer. Carcinogenicity of human papillomaviruses. *Lancet Oncol*, **6**, 204.

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**, 1057-62.

De Sanjose S, Quint WG, et al (2010). Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide. *Lancet Oncol*, **11**, 1048-56.

Eklund C, Zhou T, Dillner J. WHO Human Papillomavirus Laboratory Network (2010). Global proficiency study of human papillomavirus genotyping. *J Clin Microbiol*, **48**, 4147-55

Fattorini P, Marrubini G, Ricci U, et al (2009). Estimating the integrity of aged DNA samples by CE Electrophoresis, **30**, 3986-95.

Ferlay J, Shin HR, Bray F, et al (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*, **127**, 2893-917.

Hammouda D, Muñoz N, Herrero R, et al (2005). Cervical carcinoma in Algiers, Algeria: human papillomavirus and lifestyle risk factors. *Int J Cancer*, **113**, 483-9.

Harper DM, Franco EL, Wheeler CM, et al (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**, 1247-55.

Hsairi M, Fakfakh R, Bellaaj R, Achour N (2003). Attitude to cervical cancer screening of women residing in two regions of northern Tunisia. *Tunis Med*, **81**,721-30.

Human papillomavirus vaccines. WHO position paper (2009). *Wkly Epidemiol Rec*, **84**, 118-31.

KrennHrubec K, Mrad K, Sriha B, et al (2011). HPV types and variants among cervical cancer tumors in three regions of Tunisia. *J Med Virol*, **83**, 651-7.

Kulkarni SS , Kulkarni SS, Vastrad P P, et al (2011). Prevalence and distribution of high risk human papillomavirus. (HPV) Types 16 and 18 in carcinoma of cervix, saliva of patients with oral squamous cell carcinoma and in the general population in karnataka, India. *Asian Pac J Cancer Prev*, **12**, 645-8

Lazzar BG, Hajjem S, Aounallah-Skhiri H, Achour N, Hsairi M (2011). Mortality from cancer in Tunisia: calculating years of life lost. *Sante Publique*, **23**, 31-40

Lee SH (2012). Guidelines for the use of molecular tests for the detection and genotyping of human papilloma virus from clinical specimens. *Methods Mol Biol*, **903**, 65-101.

Longy M, Duboue B, Soubeyran P, Moynet D (1997). Method for the purification of tissue DNA suitable for PCR after fixation with Bouin's fluid. Uses and limitations in microsatellite typing. *Diagn Mol Pathol*, **6**, 167-73.

Mc Sherry EA, Mc Goldrick A, Kay EW (2007). Formalin-fixed paraffin-embedded clinical tissues show spurious copy number changes in array-CGH profiles. *Clin Gen*, **72**, 441-7.

Missaoui N, Hmissa S, Trabelsi A, et al (2010). Prevalence of HPV infection in precancerous and cancerous lesions of the uterine cervix in Tunisia. *Ann Biol Clin*, **68**, 297-303.

Muñoz N, Bosch FX, Castellsagué X, et al (2004).Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer*, **111**, 278-85.

Njah M, Ben Ahmed S, Marzouki M (1994). Cancer of the breast and uterine cervix: knowledge level and preventive practices in a segment of the Tunisian population. *Sante*, **4**, 299-302.

Paavonen J, Naud P, Salmeron J, et al (2009). Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. *Lancet*, **374**, 301-14.

Shen XH, Liu SH (2013). Human papillomavirus genotypes associated with mucopurulent cervicitis and cervical cancer in hangzhou, China. *Asian Pacific J Cancer Prev*, **14**, 3603-6.

Sjoeborg KD, Tropé A, Lie AK, et al (2010). HPV genotype distribution according to severity of cervical neoplasia. *Gynecol Oncol*, **118**, 29-34.

Villa LL, Costa RL, Petta CA, et al (2006). High sustained efficacy of a prophylactic quadrivalent human papillomavirus type 6:11:16:18 L1 virus like particle vaccine through 5 years of follow up. *Br J Cancer*, **95**, 1459-66.

Walboomers JM, Jacobs MV, Manos MM,, et al (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Patho*, **189**, 12-9.

Webersinke C, Doppler S, Roithmeier F, Stummvoll W, Silye RJ (2013). Cervical biopsies and cytological smears - A comparison of sample materials in HPV diagnostics. *Clin Virol*, **56**, 69-71.

World Health Organization (2009): Human papillomavirus laboratory manual. 1st eds Unger E. R, Dillner J, Zhou T . Geneva, Switzerland..