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ORIGINAL ARTICLE

Prevalence of HPV genotypes in cervical adenocarcinoma: a study in Greek women

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Summary

Purpose: *To study the prevalence of human papillomavirus (HPV) genotypes among cervical adenocarcinomas in Greek women.*

Methods: *The study group comprised 78 adenocarcinoma cases (20 in situ and 58 invasive). HPV DNA was amplified using polymerase chain reaction (PCR) and HPV genotypes were identified by reverse hybridization.*

Results: *There was a high prevalence of HPV infection both for in situ (95%) or invasive (94.83%) adenocarcinomas, comprising also cancers of unusual morphology. HPV 16 was the commonest strain (N=57, 73.08%) followed by HPV 18 (N=28, 35.90%). Interestingly, 13 cases (16.67%) were also HPV 52 positive (as co-infection with HPV 16 or 18). All other strains with the exception of HPV 66 were found only as co-infections. No significant age difference was noted in terms of any HPV strain positivity.*

Conclusions: *HPV DNA was found in the large majority of cervical adenocarcinomas. As opposed to other studies, HPV 52 was the third most commonly encountered strain after HPV 16 and HPV 18. The above findings would probably be of help in decision making concerning vaccination policy for the prevention of HPV infection in Greece.*

Key words: *adenocarcinoma, cervix, Greece, HPV, PCR*

Introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females worldwide, accounting for 9% (529,800) of the total new cancer cases and 8% (275,100) of the total cancer deaths among females [1]. Despite the significant reduction in the incidence of invasive squamous cell carcinoma, due to the introduction of the Pap smear, a rise in the incidence of cervical adenocarcinoma has been recently observed [2]. It has long been established that HPV is the central cause of cervical cancer [3], squamous cell carcinomas almost always harboring an oncogenic HPV. On the other hand, studies on cervical adenocarcinomas revealed a diminished prevalence of HPV infection which was mostly encountered in unusual morphological types of carcinomas, specifically clear cell, serous, minimal deviation adenocarcinomas and those of gastric or intestinal phenotype [4,5]. Moreover, factors such as the age of the patients and HPV type specific prevalence related to the geographical region of the specimen have been thoroughly studied [5,6].

In the present study, we sought to determine the prevalence and distribution of HPV genotypes in intraepithelial and invasive cervical adenocar-

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cinomas of Greek women by conducting a retrospective, hospital-based study. Given the recent development and introduction of HPV vaccines our goal was to determine whether their protective role would encompass the whole spectrum of cervical glandular neoplasia or whether some unusual subtypes will not be prevented by current HPV vaccines.

Methods

Archival material from 148 women who were diagnosed with cervical adenocarcinoma between the years 2007 to 2015 was selected from the files of the Pathology Department of "Iaso" Women's Hospital, Athens, Greece. These cases comprised cervical swabs, small biopsy specimen, loop/conization material and trachelectomy or hysterectomy specimen. The respective slides were reviewed by two pathologist specialized in Gynecological pathology (P.Y, I.M). Cases featuring both glandular and squamous neoplasia and a neuroendocrine component were excluded from the study thereafter our final study group included 78 cases. Of these, 20 were *in situ* and 58 invasive adenocarcinomas. The histologic subtype of the invasive cancers was as follows: of usual type (N=44), villoglandular (N=5), serous (N=3), clear cell (N=2), minimal deviation (N=2), mucinous (N=1) and adenoid basal (N=1). Moreover, 7 benign endocervical polyps comprising only glandular epithelium were used as controls.

The study protocol was approved by the Ethics Committee of the Hospital and informed consent was obtained from all patients.

Tissue dissection and DNA preparation

In order to prevent case-to-case HPV DNA contamination and ensure that lesional tissue was sectioned for PCR analysis, the cutting of the paraffin blocks followed a routine called a "sandwich technique". In this technique, the first 4 μm slide was used for hematoxylin-eosin (H&E) staining, then two 4 μm tissue sections were cut and collected to a 1.5 ml tube, finally again a 4 μm slide was cut for H&E staining to confirm that the diagnostic sample was present in the tube collected for PCR.

Appropriate positive and negative controls were incorporated during DNA preparation and subsequent testing to monitor for a possibility of cross-contamination.

HPV analysis

The extraction of HPV DNA was performed with the Amplilute Media Extraction Kit protocol (Roche Molecular Systems) in the case of cervical cells collected in cobas® PCR Cell Collection Media or PreservCyt® Solution Liquid. For formalin-fixed and paraffin-embedded tumor tissues (FFPET Sections) cobas® DNA Sample Preparation Kit (Roche Molecular Systems), a generic manual specimen preparation based on nucleic acid binding to glass fibers [7,8] was used .

Concerning the latter method, one deparaffinized (with the use of xylene) 4 μm section of an FFPET specimen, which was prepared with the previously described "sandwich technique", was lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released genomic DNA from DNases. Subsequently, isopropanol was added to the lysis mixture that was then centrifuged through a column with a glass fiber filter insert. During centrifugation, the genomic DNA was bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, were removed by centrifugation. The adsorbed nucleic acids were washed and then eluted with an aqueous solution. The amount of genomic DNA was spectrophotometrically determined and adjusted to a fixed concentration to be added to the amplification/detection mixture.

Broad-spectrum HPV DNA amplification was then performed using a short-PCR-fragment assay. As it was mentioned, the most conserved region in the HPV genome is the L1 region, and several consensus PCR primer sets have been previously described for this region [9]. Examples are the GP5+/6+ [10], MY09/11 [11] and PGMY [12] primer sets. The primer set used in the INNO-LiPA HPV Genotyping Extra II Amp amplifies a 65-bp region in the L1 open reading frame [13].This primer set is an upgrade version of the "SPF10" primer set. HPV-positive specimens were typed using reverse hybridization Line Probe Assay which enables the detection of at least 54 HPV types [14] and the LiPA strips were manually interpreted using the reference guide provided.

Statistics

According to their histological subtype, all cervical adenocarcinoma cases were grouped as in situ (Group A), or invasive, being sub-classified as those of usual type (Group B) and those of rare subtypes (Group C). The latter group comprised cases of villoglandular, serous, clear cell, mucinous, adenoid basal and minimal deviation adenocarcinomas, Differences of mean age between groups or between cases classified as positive or negative according to a certain HPV strain, were assessed by the Mahn-Whitney U test. Pairwise differences between groups in terms of HPV positivity rates were assessed by proper x^2 test. Every observation with $p<0.05$ was considered significant.

Results

Our results indicate a high prevalence of HPV infection among cervical adenocarcinomas in the Greek population, both *in situ* (95%) and invasive (94.83%).

Histologic subtypes and tumor HPV status

Seventy eight patients (N=78) diagnosed with adenocarcinoma of the cervix were enrolled in the current study. Apart from in situ (N=20, 25.64%) and usual type invasive (N=44, 56.41%) adenocarcinomas of the cervix, 5 viloglandular (6.41%), 3 serous (3.84%), 2 clear cell (2.56%), 2 minimal deviation (2.56%), 1 case of mucinous carcinoma (1.28%) and 1 case of adenoid basal adenocarcinoma (1.28) were also encountered.

All adenocarcinoma cases were assessed for the presence of specific HPV strains. It was found that HPV 16 was the commonest strain (N=57, 73.08%) followed by HPV 18 (N=28, 35.90%). Interestingly, 13 cases (16.67%) were also HPV 52 positive (as co-infection with HPV 16 or 18), while 5 cases (6.41%) were positive for HPV 45 (as co-infection mostly with HPV 16), 5 cases (6.41%) were positive for HPV 53 and 4 cases (5.13%) were positive for HPV 51 (Table 1). Apart from these strains, several others were identified in smaller rates (Table 2) as co-infections with other strains. In 2 cases the experimental procedure was marked as failure.

There was no significant correlation of patients' age with the histologic subtypes and tumor HPV status

Pairwise comparisons did not reveal any significant age difference between the groups A, B and C. No significant age difference was noted in terms of any HPV strain positivity. Finally, HPV positivity rates did not differ significantly between the groups of the study.

A high prevalence of HPV positivity was found in the control samples

As shown in Table 3, 5 out of the 7 controls were positive for HPV, either as single infection or co-infection with strains that belonged to the high-risk or possible high-risk group. HPV 16 or 18 were not encountered in the control group. The age of the controls ranged from 35 to 66 years (mean=47).

Discussion

To our knowledge this is the first study on the molecular epidemiology of HPV infection in cervical adenocarcinoma in Greek women performed mainly on formalin fixed and paraffin embedded histological material. Despite the relatively small number of cases, several factors strengthen our results. The study population is relatively homogeneous since it derives from a large private wom-

Table 1. Distribution of HPV strains within each histological subtype

Histological subtypes (N=78)	*HPV16 (HR) N (%)*	*HPV18 (HR) N (%)*	*HPV52 (HR) N (%)*	*HPV45 (HR) N (%)*	*HPV6 (LR) N (%)*	*HPV53 (pHR) N (%)*	*HPV51 (HR) N (%)*	*Other HPV types (39, 66, 73, 31, 33, 58, 62, 68) N (%)*	*HPV (-) N (%)*
In situ (N=20)	14 (70)	6 (30)	2 (10)	2 (10)	2 (10)	1 (5)	2 (10)	3 (15)	1 (5)
Invasive (N=58)	43 (74)	22 (38)	11 (19)	3 (5.2)	5 (8.6)	4 (6.9)	2 (3.4)	7 (12.07)	2 (3.4)
Usual type (N=44)	33 (75)	18 (41)	11 (25)	1 (2.3)	4 (9.1)	3 (6.8)	1 (2.3)	3 (6.81)	1 (2.3)
Rare type (N=14)	10 (71.4)	4 (28.5)	0 (0)	2 (14.3)	1 (7.1)	1 (7.1)	1 (7.1)	4 (28.57)	1 (7.1)
viloglandularm (N=5)	3	2	0	1	0	0	0	3	0
serous (N=3)	3	2	0	0	0	0	0	0	0
mucinous (N=1)	1	0	0	0	1	1	0	0	0
clear cell (N=2)	1	0	0	0	0	0	0	0	1
adenoid basal (N=1)	1	0	0	1	0	0	0	0	0
minimal deviation (N =2)	1	0	0	0	0	0	1	1	0
Total	57 (73.08)	28 (35.90)	13 (16.67)	5 (6.41)	7 (8.97)	5 (6.41)	4 (5.13)	10 (12.82)	3 (38.46)

HR: high-risk strain, LR: low-risk strain, pHR: probably high-risk strain

Table 2. Distribution of HPV strains within the sample of study

HPV strain	*Single infection*	*Coexisting with other strains*	*Total*	
			N	*%*
16	28	29	57	73.08
18	12	16	28	35.90
52	0	13	13	16.67
6	0	7	7	8.98
45	0	5	5	6.41
53	0	5	5	6.41
51	0	4	4	5.13
39	0	2	2	2.56
66	1	1	2	2.56
73	0	2	2	2.56
31	0	1	1	1.28
33	0	1	1	1.28
58	0	1	1	1.28
62	0	1	1	1.28
68	0	1	1	1.28

Table 3. Distribution of HPV strains within the control samples

Control number	*Age (years)*	*Histology*	*INNO-LiPA**
1	56	endocervical glandular polyp	68
2	42	endocervical glandular polyp	59, 68
3	66	endocervical glandular polyp	58
4	35	endocervical glandular polyp	HPV negative
5	41	endocervical glandular polyp	HPV negative
6	40	endocervical glandular polyp	52, 68
7	49	endocervical glandular polyp	66

*INNO-LiPA HPV Genotyping Extra II (Fujirebio Europe N.V., Belgium)

en's hospital where most usually Greek women are addressed. Our paraffin blocks were well preserved and dated from 2007 to 2015, minimizing the possibility of technical artifacts due to tissue degradation as can be encountered with longer archived material [19]. Finally, as already stated in the material section, cases were selected using rigorous criteria, therefore excluding coexisting squamous or neuroendocrine neoplasia.

Our results indicate a high prevalence of HPV infection among cervical adenocarcinomas in the Greek female population, both in situ (95%) and invasive (94.83%). The above prevalence is higher than the one presented worldwide ranging from 65% [19] to 85% [6] but is close to percentages derived from publications focusing on more homogeneous ethnic groups such as the Korean or Scottish population [20,21].

In our series of cases, HPV 16 was by far the most commonly observed strain followed by HPV 18, both being mostly found as single infections, a finding which is in agreement with most studies [5,15,19,22]. Yet, HPV 52 was the third most commonly encountered strain with a prevalence of 16.6%, always as a co-infection, a finding that might represent a population-based difference. Indeed, despite the fact that HPV 52 has been considered as belonging to the most carcinogenic strains, its worldwide prevalence in all histological types of cervical carcinoma is relatively low ranging from 2% in Europe to 4% in Asia [19,23]. Moreover, when stratifying by histological type there was no identifiable case of cervical adenocarcinoma, positive for HPV 52. In our study, HPV 45 which is considered by most researchers as of high prevalence in cervical adenocarcinoma, represented only a small subset of patients with percentages close to those obtained for other less common strains as seen in Table 1.

Given our results one could hypothesize that, with the exception of HPV 16 and HPV 18, none of the other HPV strains detected in our study (52, 45, 53, 51, 39, 73, 31, 33, 58, 62 and 68) were capable to induce carcinogenesis as a single infection. Indeed, in a large scale population-based study comprising pooled data from three continents, no patients with invasive cervical adenocarcinoma were found to be infected with some of the above types, specifically types 39, 52, 56, 68 or 73, and only one patient was infected with HPV 31. The authors concluded that the above strains, although classified as high-risk when studying squamous cell carcinoma could not be confirmed as high-risk types for adenocarcinoma [24]. Therefore, their role in the genesis and progress of cervical adenocarcinoma should probably be the subject of epidemiological research.

Of interest is the single infection by HPV 66 encountered in one of our cases representing a minimal deviation adenocarcinoma. Although

HPV 66 shares many similarities of the nucleotide sequence of E6 and E7 with HPV 16, it is rarely found in cervical carcinomas [25]. In a retrospective cross-sectional worldwide study it was detected in <1% of squamous cell cervical carcinomas and in none of the 470 cases of cervical adenocarcinomas [19]. Recently, the Working Group of the World Health Organization International Agency for Research on Cancer (IARC) proceeded to a re-classification of HPV 66 from carcinogenic to possible carcinogenic since "it was found as a single infection in cancers with extreme rarity, well below the threshold of possible confounding and misclassification" [23]. As far as our case is concerned, we would just want to emphasize the rare occurrence of an HPV 66 single infection in a case of a rare variant of cervical adenocarcinoma which is generally considered as not being HPV related.

Our study revealed some more exclusive characteristics. It was shown that positive results have also been obtained in 5 out of 7 of our benign samples but also in all but one of our cervical adenocarcinomas of unusual morphological type, including the 2 cases of minimal deviation adenocarcinomas which are considered by several investigators as not being related to HPV infection [4]. One could argue that the above findings might represent false positive results and of course this is a possibility. Yet, our tissue handling procedure minimizing the possibility of case-to-case contamination and the rejection from our study of all cases with coexisting squamous neoplasia of any degree renders this possibility rather inexistent. Do we confront some kind of population related oncogenic or non-oncogenic HPV strains?

When trying to respond the above dilemma, several individual thoughts arise. As far as our normal endocervical samples are concerned, most of the HPV strains that were identified (68, 58, 52 and 66) belong to the already mentioned group of viruses that might not represent high-risk strains when considering adenocarcinomas [24]. The above observation strengthens the hypothesis that despite the fact that the aforementioned strains were classified in the initial epidemiological work by Munoz et al. [26] in the sub-category of high-risk HPV strains the whole work was based on squamous neoplastic lesions. One could speculate that in a setting of glandular neoplasia, viral integration and neoplastic transformation might follow different pathogenetic pathways. The same could apply to one of our benign cases which presented a co-infection with HPV strains 68 and 59.

Our findings on the normal endocervical samples raise the question of the carcinogenicity of individual HPV types other than HPV 16 and HPV 18. A novel technique in the field of HPV research studies, Laser Capture Microdissection (LCM), has already shown that, concerning squamous neoplastic lesions, only one high-risk HPV type is found in cancer cells or in a defined area of cervical intraepithelial neoplasia (CIN), even if a whole-tissue specimen is positive for multiple HPV types [15,27]. However, these results were applied to CIN lesions, where there is a distinct area of normal and CIN epithelium of any degree which can be clearly defined specifically by expert pathologists. Concerning glandular epithelia as a morphologic continuum does not exist. Nevertheless, LCM could probably be used to isolate both morphologically benign glandular cells and neoplastic glandular epithelium in order to identify whether or not such distinct areas are characterized by different HPV strains.

Regarding the nearly total identification of HPV infection in most unusual types of cervical adenocarcinomas that were enrolled in our study, a finding which is in contrast to the results obtained by other investigators [4], we could speculate that it might represent a population-based difference or to the use by other researchers of less sensitive tests for HPV DNA detection [4,28] or even to the interference of other factors such as inhibitors of polymerase or disruption of viral integration [20].

A number of more findings need to be underlined. Our statistical analysis did not reveal any significant differences in the strain of HPV involved in the infection among patients with *in situ* (AIS) or invasive adenocarcinoma, indicating that both conditions might have a close pathogenetic association. Apparently, the acquisition of an invasive phenotype does not seem to be related to specific properties of a particular HPV strain but to the activation of molecular pathways that lead to the accumulation of mutational defects and to the induction of a more aggressive phenotype.

Pairwise comparisons did not reveal any significant age difference between the different histological subgroups. Nevertheless, when studying by case, our unique case of intestinal-type endocervical adenocarcinoma was encountered in an elderly woman, a finding which is in accordance with the results of a recent study [29]. As far as the analysis of the median age of the patients in relation to distinct HPV strains is concerned,

no statistically significant conclusions could be drawn. Apparently, this finding, as opposed to squamous neoplastic lesions for which a substantial fraction of cervical cancers is associated with other high-risk HPV types [30], is related to the fact that in our series all other HPV strains except HPV 16 and HPV 18 were found as co-infections.

All the above remarks highlight important differences in the prevalence of specific HPV strains in a small cohort of Greek women. As opposed to studies from other ethnic groups a high prevalence of HPV infection was also found in the group of adenocarcinomas of unusual morphological type. For all three groups under evaluation HPV 16 was the most common type followed by HPV 18 and HPV 52 while HPV 45 and HPV 53 were equally represented as the fourth more common strains. Yet, the latter three strains were always found as a co-infection with either HPV 16 or HPV 18. The above findings would probably be of help in the decision-making concerning vaccination policy for the prevention of HPV infection in Greece.

Authors'contribution

A.C.: Conceived the idea, analyzed cases, wrote the paper

G.K.: Participated in analyzing the cases

T.V.: Performed the statistics

P.Y.: Diagnosed cases, reviewed the slides

I.M.: Diagnosed cases, reviewed the slides

E.P.: Diagnosed cases

K.P.: Conceived the idea, diagnosed cases, reviewed the paper

Conflict of interests

The authors declare no confict of interests.

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