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Presence of high-risk human papillomavirus sequences in breast cancer tissues and association with histopathological characteristics

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Abstract

Objective: To examine the presence of human papillomavirus (HPV) in breast cancer tissues.

Design and methods: Four different PCR methods for detection and verification of genital HPVs were applied in frozen breast cancer specimens. Tumors were also evaluated for various histopathological parameters.

Results: Seventeen samples out of 107 tested positive (15.9%). HPV RFLP typing identified a total of 21 high-risk viruses: fourteen HPV 16 (67% of all detected HPV types), three HPV 59, two HPV 58, one HPV 73 and one HPV 82 (one sample with double infection and two samples with triple infection). Breast cancer patients harboring high-risk HPV DNA sequences in their tumor were younger than the rest of the patients. Furthermore, they were less estrogen-receptor-positive and more proliferative as observed in the corresponding indices: Ki-67 staining, S-phase/proliferative fractions and percentage of cells with DNA content over 5C.

Conclusion: The presence of high-risk HPV DNA sequences in the breast cancer tissues studied was verified, and a possible association with acceleration of malignancy was examined.

Keywords: HPV; Breast cancer; PCR; RFLP; Tumor markers; Histopathology

Introduction

Mucosal human papillomaviruses (HPV) are small double-stranded DNA viruses that infect mainly anogenital epithelium [1]. The majority of these sexually transmitted genital HPV types are considered high risk because they possess at least three proteins E5, E6 and E7 with growth-stimulating and transforming properties [2]. It has been estimated that at least 99.7% of cervical carcinomas worldwide [3] and 50% of head and neck squamous cell carcinomas [4] are due to HPV. It is therefore worrisome that rates of HPV infection appear to be rapidly increasing, urging for an efficient vaccination program [1].

However, the role of HPV infection in other than the aforementioned cancers has not been established unequivocally. Breast cancer is the leading female cancer worldwide and the third in row in terms of mortality after lung and colon cancer. Most studies looking at infection and breast cancer have examined the role of other viruses like murine mammary tumor virus, simian virus 40 and Epstein–Barr virus in breast cancer etiology [4,5]. It has been proven though, in vitro, that the most efficient and reproducible model of human mammary epithelial cell immortalization is the expression of high-risk HPV oncogenes E6 and E7 [6].

Abbreviations: ASCUS, atypical squamous cells of unknown significance; CIN I–III, cervical intraepithelial neoplasia, stages I–III; ER, estrogen receptor; PgR, progesterone receptor; >5C DNA content, cells containing over 5 chromosome haploid sets, pentaploid; IHC, immunohistochemistry; QIC, Quantitative Immuno Cytochemistry score; PF, proliferative fraction; RFLP, restriction fragment length polymorphism; SSP-PCR, sequence-specific-polymerase chain reaction.

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Albeit discordant results have been obtained from studies looking for HPV DNA at breast cancer specimens with molecular techniques [7–10]. All of the above studies were performed in a limited number of paraffin-embedded breast tumors, and few of them have addressed the effects of HPV infection in various histopathological characteristics in the breast tissue (mostly looked at the presence of estrogen and progesterone receptors). Furthermore, between the HPV-positive studies, there is discrepancy as far as it concerns which types are present (low or high risk) and to the suggested route of transmission (hematogenous or external).

In the present study, we have gathered a substantial number of frozen breast cancer specimens ($n=107$) and tried to detect and verify HPV viral DNA by four different PCR methods looking at different areas in the HPV genome. HPV type was identified by restriction-fragment polymorphism (RFLP) analysis. Finally, we examined the association of the detected HPV types with the majority of various prognostic factors more or less commonly employed for breast cancer behavior assessment: histological parameters (histology, grade, lymph node involvement), hormone receptors content, p53 and c-erbB2 status, Ki-67 staining and DNA ploidy evaluation.

Materials and methods

Patients and histopathological characteristics

Consecutive breast cancer patients of Mitera Maternity and Surgical Center were included in the study after obtaining informed consent that allowed use of their biological materials for investigational purposes ($n=107$). The sole criterion was the availability of enough material to perform histology and routine immunohistochemistry for estrogen and progesterone receptors besides what is needed for DNA extraction, p53, Ki-67 and c-erbB2 IHC and ploidy tests (tumor size ≥ 1 cm). The image cytometer CAS200 (Becton Dickinson, USA) was used in order to perform DNA ploidy measurements in imprints of at least 200 Feulgen-stained tumor nuclei (ploidy status, DNA index, S-phase fraction, proliferative (G_2+M) fraction, fraction of cells with DNA content >5C). The same equipment was used to assess in a quantitative way levels of estrogen and progesterone receptors (Quantitative Immuno Cytochemistry score, QIC=positive area % ×positive stain intensity %/10) and that of the proliferation marker Ki-67 and nuclear p53 accumulation (positive area %) [11]. IHC for c-erbB2 membrane overexpression was also performed (microscopic interpretation). Reagents and evaluation for the above biological parameters were performed as described previously [12].

DNA extraction and HPV detection

Depending on the cellularity, 2–3 slides of 5-μm-thick frozen sections from breast tumor specimens were scraped with a separate sterile razor for each sample, collected in an Eppendorf vial along with PBS and centrifuged at 3000 rpm for 10 min. The wash was repeated for another time, and then the QIAamp DNA Mini Kit was used for DNA extraction (QIAGEN, Germany). DNA integrity for each sample was assessed by PCR amplification of b-globin gene with PC04 and GH020 primers [13]. For genital HPV DNA detection, the two most popular worldwide consensus PCR reactions were used: the MY system [13] and the GP+ system [14] both amplifying regions of L1 HPV gene. All PCR reactions were performed in the PTC-200 DNA Engine (MJ Research, USA) with a dual thermal block at 1.5 mM final Mg^{+2} concentration under the same cycling program: after an initial denaturation step of 4 min at 94°C, 40 cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min elongation at 72°C and finally 10 min at 72°C (for the GP+ system, we used 40°C for annealing and 2 mM final Mg concentration). QIAGEN Master Mix (2×) was used along with 3 μl of sample or control DNA (total volume: 50 μl). Appropriate controls included either H_2O (blank reaction) or DNA-negative for HPV either positive for HPV 6 DNA from a patient with flat condyloma or HPV 31 DNA from a patient with cervical cancer that were well characterized in another study [15]. All necessary standard precautions were observed in order to avoid contamination through PCR carry-over. Primers were synthesized at FORTH, Greece, and their characteristics appear in Table 1. PCR products were run in 1.5% 1× SB agarose gels. These gels are made in a cheaper, low-molarity medium that was proven to provide the same quality of separation for DNA fragments as TBE gels in just 10 min [16].

Table 1
Sequences of primers used in this study, their location in the HPV genome and corresponding PCR product length

Name	Oligonucleotide sequence (5′–3′)	Use	Gene	bp	GenBank location
MY11[a]	GCMCAGGGWCATAAYAATGG	Forward	L1	450	6583–6602[b]
MY09[a]	CGTCCMARRGGAWACTGATC	Reverse	L1		7015–7034[b]
GP5+	TTTGTTACTGTGGTAGATACTAC	Forward	L1	150	6624–6649[b]
GP6+	GAAAAATAAACTGTAAATCATATTC	Reverse	L1		6719–6746[b]
pU-H	TGTCAAAAACCGTTGTGTCC	Forward	E6	238	419–438[b]
pU-R	GAGCTGTCGCTTAATTGCTC	Reverse	E7		637–656[b]
pU-L	TGCTAATTCGGTGCTACCTG	Forward	E6	228	400–419[c]
HPV 16F	GTGGACCGGTCGATGTATGT	Forward	E6	93	496–515[b]
HPV 16R	CATGCAATGTAGGTGTATCT	Reverse	E6		570–589[b]
HPV 6/11F	ATGTTATGGCAGCACAGTTA	Forward	E4	306	3280–3299[c]
HPV 6/11R	TTGCACTATAGGCGTAGCTG	Reverse	E4		3570–3589[c]

[a] Degenerate bases: M=A+C, W=A+T, Y=C+T, R=A+G.
[b] HPV 16, GeneBank accession number K02718.
[c] HPV 6, GeneBank accession number X00203.

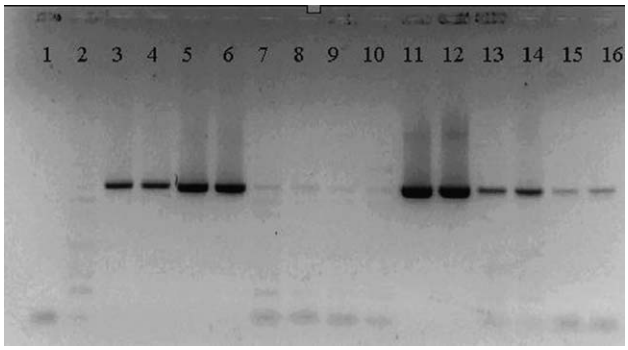

Fig. 1. Agarose gel electrophoresis of HPV MY-PCR reactions from five cervicovaginal samples (lanes 3–12) and two positive breast cancer tissues (lanes 13–16) in duplicate, run alongside a blank and a negative sample in lanes 1 and 2 (positive sample in the upper part of the gel, not shown).

HPV RFLP typing

In case of a positive sample in the MY-PCR, reactions were performed again in duplicate, mixed and their product was subjected to restriction fragment polymorphism analysis: 13 μl of PCR product plus 1.5 μl restriction buffer NEB 2 and 0.5 μl of each of these restriction enzymes: *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, *Pst*I and *Rsa*I (New England Biolabs, USA) in separate tubes. Incubations lasted 4 h at 37°C and were analyzed subsequently in a 2% Nusieve 1:1 agarose gel as previously reported [17]. Assignment of a found HPV type to a particular risk category was done according to the most recent epidemiological study [18].

Verification of HPV presence and type

Two other PCR-based methods were used for the positive samples in order to verify HPV presence and type. The first method was a set of two-reaction type-specific PCR-SSP reactions with high sensitivity either for HPV type 16 or for HPV types 6/11 that amplify E6 and E4 regions in the HPV genome respectively [15]. The second is a set of two consensus reactions with a common reverse primer (pU-R) assessing a region between E6 and E7 genes [19]: by using the first forward primer, low-risk HPV types 6 and 11 are detected (pU-L PCR), while by using the other forward primer, high-risk HPV types 16, 18, 31, 33, 52 and 58 are detected (and according to our experience, types 35 and 59 too, pU-H PCR). PCR programs were as in MY-PCR reaction, and the characteristics of the primers are shown in Table 1. Positive controls containing HPV 6 and HPV 16 were obtained from the same source [15].

Statistics

Data analysis was carried out with SPSS 11.0 for Windows (SPSS Inc., USA). The Mann–Whitney test for non-normally distributed between two independent groups was used for all histopathological parameters. In the case of grade and ploidy, nominal values were grouped in two different categories.

Results

HPV detection

HPV DNA was detected in 17 out of 107 frozen breast cancer tissues (15.9%) by MY-PCR. The ~450 bp PCR product signals (size dependent on HPV type) obtained by the above method in the breast cancer tissues were quite weaker in the UV-illuminated gels than the corresponding ones we get from our routine gynecological cervicovaginal samples (Fig. 1). In eleven of these samples, a ~150 bp band was also obtained from the other consensus GP+ system (Table 2). No sample was positive in the GP+ system and negative in the MY system. There is a 65% (11/17) concordance between the two popular consensus systems, analogous with what is seen in our routine HPV DNA detection. This is due to the fact that both PCR reactions are amplifying the same region in the L1 gene that is conserved between various HPV genotypes but still with some heterogeneity. The GP+ primers are internal to the MY primers, however, we chose not to try the contamination-prone nested PCR approach in such a debate-stirring research subject. In all PCR methods (including the verification methods), reactions with an appropriate blank, a negative and a positive control were always included, and the expected results were obtained. Normal benign breast tissue unfortunately was not available to test however tissues from breast fibroadenomas and reduction mammoplasty in another study were found negative for the presence of HPV virus [9].

HPV RFLP typing

In all HPV-positive cases by MY-PCR ($n=17$), RFLP typing was performed with either five or six restriction enzymes. Enzyme digests were run in high-resolution gels, and results are recorded in Table 2. In one of the MY-PCR positive samples, we could not assign a type because the reaction was weak (R375). In the rest of

Table 2
Results from various PCR reactions and HPV RFLP typing for all the samples being positive in the MY-PCR reaction ($n=17$)

Tumor ID	Age	Histology	MY-PCR	GP+ PCR	SSP-16 PCR	pU-H-PCR	HPV type
R375	57	DC[a]	wk+	−	−	−	−
R377	30	DC	+	+	−	−	16
R405	34	DC	wk+	−	+	wk+	16
R415	34	DC	+	+	−	+	16
R416	38	DC	+	−	+	+	16
R419	37	DC	+	wk+	−	−	16
R532	35	DC	+	+	−	+	16
R536	43	DC	+	+	−	−	16
R563	35	DC	+	+	−	+	16
R567	38	DC	+	+	−	+	16
R573	38	DC	+	+	+	−	16
R717	52	Apocrine	+	−	−	+	58+59
R721	56	DC	wk+	+	−	−	59
R739	67	DC	+	+	+	−	58+59+16
R785	51	DC	+	−	−	+	16
R804	41	DC	wk+	−	−	+	16
R814	48	Papillary	+	+	−	−	16+73+82

[a] DC = invasive ductal carcinoma.

the samples, 21 HPV types were identified: fourteen HPV 16 (67% of all detected HPV types), three HPV 59 (14%), two HPV 58 (10%), one HPV 73 (5%) and one HPV 82 (5%). All of the above HPV types are categorized as high-risk types due to their oncogenic potential [18]. Two triple- and one double-infection cases were found. The RFLP analysis of the double infection found in sample R717 can be seen in the Nusieve agarose gel in Fig. 2.

Verification of HPV presence and type

Due to the HPV types assigned in the MY-PCR positive samples, we chose to perform another two PCR methods to verify the presence and type of HPV in these 17 samples (Table 2). In the pU-H PCR method, the expected 238 bp band of the E6/E7 region of certain high-risk HPV types was seen in 10 samples (59% concordance with MY-PCR). No positive result was obtained from the pU-L PCR method that detects low-risk HPV types 6 and 11 as expected from the RFLP-typed samples. In the SSP-PCR method for HPV 16, the expected 93 bp band of the E6 region was seen in 4 out of 14 samples typed as HPV 16 (29% concordance with MY-PCR). No positive result was obtained for the SSP-PCR method for low-risk HPV 6/11 types.

Histopathological characteristics

Breast cancer patients found positive for the presence of high-risk HPV DNA sequences in their tumor were 15 years younger than the rest of the patients (median, 25th–75th percentile: 38 (35–51) years compared with 53 (44–63), $P=0.001$, Mann–Whitney). As can be seen in Table 3, tumors harboring HPV sequences in their genome were less ER-positive and more proliferative as observed in the corresponding indices: Ki-67 staining, S-phase fraction, proliferative fraction and percentage of cells with DNA content over 5C (all statistically significant, $P<0.05$). The above observation was reflected also in the histomorphological parameter of grade: 70.6% of the HPV containing tumors were classified as grade III compared with 33.3% of the rest of the tumors ($P=0.005$). Positivity for immunohistochemical p53 nuclear accumulation was not different between the two groups. No other parameter presented with statistically significant difference in the HPV DNA containing group: PgR QIC and c-erbB2 positivity were practically identical between the two groups. Furthermore, histology types were not different from the control group with the ductal pattern dominating. The histopathological characteristics of the breast cancer patients tested negative for the presence of HPV DNA sequences were not different from any other group of breast cancer patients analyzed under the same methods by our group.

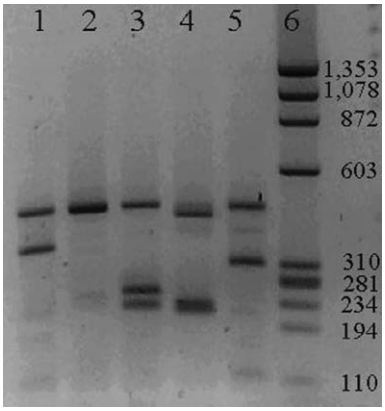

Fig. 2. RFLP analysis of the MY-PCR product for tumor sample R717 showing double infection with two types: HPV 58 and HPV 59. From left to right (lanes 1–6): *Dde*I, *Hae*III, *Hin*fI, *Pst*I and *Rsa*I digests and φχ-174 *Hae*III molecular marker (MWs are shown in the right side).

Table 3
Comparison of histopathological parameters between breast tumors harboring high-risk HPV DNA sequences ($n=17$) and those that they do not ($n=90$)

Parameter	High-risk HPV-positive	HPV-negative	*P* value
Age	38 (35–51)	53 (44–63)	0.001*
Grade (grade III %)	70.6	33.3	0.005*
Histology (ductal %)	88.2	73.4	0.66
Lymph node (pos %)	43.8	58.7	0.28
ER (QIC)	4.0 (1.0–174)	161 (22.8–273)	0.009*
PgR (QIC)	26.0 (2.0–129)	34.5 (3.0–136)	0.92
p53 (pos area %)	5.1 (0.6–35.5)	1.2 (0.4–12.5)	0.27
Ki-67 (pos area %)	28.6 (20.4–48.6)	20.8 (12.7–35.8)	0.049*
c-erbB2 (pos %)	25.0	25.0	1
Ploidy (aneuploid %)	62.5	43.8	0.17
DNA index	1.74 (1.60–2.02)	1.75 (1.48–1.98)	0.65
S-phase (%)	12.0 (7.0–19.0)	8.0 (4.0–13.0)	0.029*
PF (%)	23.4 (16.0–33.0)	13.0 (4.0–23.0)	0.012*
>5C cells (%)	21.0 (11.0–29.0)	12.0 (1.0–25.5)	0.003*

Non-parametric Mann–Whitney test was used to obtain *P* values from scale values [median (25th–75th percentile range)] and nominal values in percentages.
* denotes statistical significance.

Discussion

In the present study, we detected the presence of high-risk HPV DNA sequences in 17 out of 107 frozen breast cancer specimens. This prevalence (15.9%) is lower than the reported range in analogous previous studies: 25–86% [8–10]. We identified 21 HPV viruses in these 17 HPV-positive specimens since some of them were double or triple infections, a feature common in routine HPV DNA testing as well. The majority (67%) of the identified types were HPV 16 followed by HPV 59 and HPV 58. The variety of the HPV types found and the specificity of the digests of the MY-PCR products are strong arguments against any contamination hypothesis for our findings. All these types belong to the high-risk class of HPV genotypes. This sounds quite reasonable as these are the types that possess oncogenic potential [18]. For example, in the case of cervicovaginal lavages, these types lead the progression of ASCUS-characterized cytology samples to CIN lesions and then to cervical cancer [1]. However, in the most recent of the analogous studies [10], the majority of HPV detected in breast tumor belonged to the low-risk category (HPV 11 and 6), a finding not shared by the other two studies where HPV 16 and 18 seemed to dominate [8,9]. We further investigated this by the introduction of two other highly sensitive PCR protocols (pU-L and SSP-6/11-PCRs) that were also unable to locate low-risk HPV DNA sequences in our samples. When we tried to verify

the presence and type of HPV with SSP-PCR methods, some of the reactions were not positive. An explanation might be that they are not consensus primers that allow for relaxed stringency of the annealing step in the PCR reaction. Primer sites might have been mutated or even deleted in the proliferative environment of breast malignancy and especially many years after infection where probably no selective pressure exists for their retention in the HPV genome. It is very likely therefore that what we detect is not anymore an intact HPV virus but rather remaining HPV DNA sequences.

Whether the HPV-harboring breast tumor specimens belonged to patients with CIN lesions before the initiation of breast carcinogenesis was not known in our study. HPV 16 is also the predominant type in HPV-positive cervicovaginal samples in Greece (20.4%) followed by low-risk types HPV 53 (10.7%) and HPV 6 (9.3%), while HPV 58 and 59 do not exceed 4% and types 73 and 82 are extremely rare [Kroupis C., Vourlidis N. et al., in preparation]. It is not surprising therefore that the majority of high-risk HPVs in the breast tumors in our study belong to HPV 16. The Norwegian study also showed association of the presence of HPV 16 and 18 in 19 out of 41 breast cancer patients (46%) with previously treated CIN III lesions [8]. In our study, this could be reflected by the earlier onset of the disease in the breast cancer patients with HPV related to those without the virus (15-year median difference between the two groups). Since this sexually transmitted virus affects mostly young people, a latency period for HPV might be anticipated if proven to act in breast pathology. How this virus finds the way to the breast tissue was not addressed in our study and is still a matter of debate between those supporting a hematogenic and/or lymphatic transfer [8] and those implying an external route through sexual practices [10]. The former hypothesis is supported by the presence of HPV virus in other unexpected sites such as Hodgkin's lymphoma and bronchopulmonary cancer [20].

HPV-harboring tumors are less ER-positive and more proliferative as observed in the following indices: Ki-67 staining, S-phase and proliferative fractions and >5C cells ($P<0.05$, Mann–Whitney). All of the above have been proven as objective proliferation markers especially when performed with a cytometric technique. Moreover, 70.6% of the HPV-positive breast tumors were classified as grade III, compared with 33.3% of the rest of the tumors ($P=0.005$). No other parameter was any different between the two groups of patients (p53, ploidy status, DNA index, histology classification, lymph node involvement, PgR and c-erbB2 status). Only two other studies have addressed the issue of histopathological parameters and solely for the hormone receptors in paraffin sections, where they did not observe any difference [8,9]. However, this was not performed in the same objective quantitative manner as it was executed with image cytometry in our study. Further basic studies are needed to investigate whether this addition to the proliferative potential and aggressive phenotype of the harboring tumor is due to a direct role of high-risk HPV in breast carcinogenesis or due to an interaction with host genetic components. If HPV proves to be a real threat for the breast tissue as well besides the anogenital area, then extensive prophylaxis and anti-viral treatment for its eradication must be sought in every circumstance.

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