SPANDIDOS PUBLICATIONS

Identification and characterization of human papillomavirus DNA sequences in Italian breast cancer patients by PCR and line probe assay reverse hybridization

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Received May 12, 2008; Accepted July 18, 2008

DOI: 10.3892/mmr_00000011

Abstract. Human papillomavirus (HPV) infection is known to play a fundamental role in cervical and other ano-genital human cancers. The recent identification of HPVs in human breast tumors and the immortalization of normal breast cancer cells by HPV high risk types 16 and 18 suggest that the virus could be implicated in the pathogenesis of human mammary tumors. In this study, we investigated the presence of high and low risk HPV genotypes in 30 human breast cancers of different histotypes by PCR with specific HPV primers (MY09/MY11 and GP5$^+$/GP6$^+$) and by line probe assay (LiPA) reverse hybridization. Since the only positive case (untypable HPVX$^+$) was a papillary breast carcinoma, a rare tumor variant, we analyzed a further cohort of 32 papillary cancers and found one additional HPV DNA-positive case (HPV66$^+$). Our results suggest that HPV infection is not significant in mammary tumorigenesis, with the exception of particular tumor histotypes, such as papillary cancer.

Introduction

Breast cancer is a malignant neoplasia with a high incidence in women in most countries of the western world (1). The aetiology of breast cancer remains largely unknown. Although many risk factors have been associated with the development of the disease, including hormone status, cigarette smoking and alcohol consumption, the molecular mechanisms related to breast carcinogenesis remain poorly understood (2). Hereditary factors are known to be responsible for only approximately 5-10% of all breast tumors (3,4). The identification of other factors implicated in sporadic breast carcinoma onset is therefore of great interest.

More than 100 genotypes of human papillomavirus (HPV) have been identified, although only a subset of these have carcinogenic potential (5). HPV selectively infects the epithelium of the skin and mucous membranes. Specific HPV types are associated with squamous cell carcinoma, adenocarcinoma and dysplasias of the cervix, penis, anus, vagina and vulva (6). Using current technology, HPV DNA can be detected in 95-100% of cervical cancer specimens, and is regarded as a prerequisite for disease occurence (7,8).

The hypothesis that HPV might play a role in breast carcinogenesis originated from an experimental system in which a human mammary cell line was immortalized after transfection with the full-length HPV type 16 or 18 genome (9). However, the presence of HPV in malignant tumors of the breast has been controversial.

Di Lonardo *et al* were the first to report a relationship between HPV and breast cancer, demonstrating HPV16 DNA in 29.4% of 17 breast carcinomas analyzed by PCR (10).

The presence of HPV DNA has been found in various percentages in different studies. Damin *et al* detected HPV16 and/or HPV18 DNA in approximately 25% of breast carcinomas, but none in benign breast cancer specimens (11). Similar results were obtained for HPV33 DNA in Chinese and Japanese patients with breast cancer (12,13), and in a Greek study (14). HPV18 DNA was demonstrated in 48% of malignant mammary tumors from Australian women (15).

Notably, a Norwegian study on women with a history of high-grade cervical intraepithelial neoplasia (CINIII) and breast carcinoma as a second primary neoplasia showed the presence of HPV16 DNA in 46% of breast cancer specimens, suggesting that HPV-associated cervical neoplasia might be the original site of HPV infection from which the virus is transported to the breast (all HPV16-positive breast cancers were HPV16-positive in their corresponding CINIII lesions) (16). Moreover, in another study, the axillary lymph nodes of

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Abbreviations: HPV, human papillomavirus; PCR, polymerase chain reaction; LiPA, line probe assay

Key words: human papillomavirus infection, breast tumor, breast papillary carcinoma

patients with both HPV-positive cervical and breast cancer showed the presence of the same HPV genotype, suggesting that HPV may be transported by the bloodstream (17).

In contrast, other researchers have failed to demonstrate the presence of HPV DNA in samples of breast carcinoma, even when the same methods of detection were applied (18-22). Moreover, a high percentage of low risk genotype HPV DNA was present not only in breast tumors, but also in normal tissue (23,24).

This study verified the presence and characterized the specific genotype of HPV DNA in breast carcinomas from Italian patients, with the aim of assessing the role of HPV infection in mammary tumorigenesis.

Materials and methods

Specimens. Thirty formalin-fixed paraffin-embedded samples of breast cancer were obtained from the archives of the Unit of Pathological Anatomy of S. Andrea Hospital in Vercelli. These specimens included various types of breast carcinoma randomly selected for patient age, grade and receptor expression (Table I). Afterwards, an additional 7 and 25 formalin-fixed paraffin-embedded samples of papillary breast cancer were obtained from the archives of the Unit of Pathological Anatomy of S. Andrea Hospital and from the archives of the Unit of Pathological Anatomy of S. Martino Hospital in Genoa, respectively.

H&E-stained slides from the blocks were analyzed for the presence of tumor tissue, histotype and grading by two pathologists (P.M. and G.A.). Two or three 10-μm sections from each sample were collected in sterile tubes. DNA was extracted using the High Pure PCR Template Preparation kit (Roche Molecular Biochemicals) according to the manufacturer's protocol.

PCR analysis. Genomic DNA (50-100 μg) of each sample was amplified by PCR. The quality of the DNA obtained from the fixed samples was evaluated by amplification with specific primers of a 248-bp fragment of the housekeeping ß-globin gene. The presence of HPV DNA sequences was verified by amplification with two specific sets of primers, MY09/MY11 and GP5$^+$/GP6$^+$, generating a 450-bp and a 150-bp fragment, respectively (25).

ß-globin PCR was carried out in a reaction volume of 50 μl containing 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer and 0.05 U/μl Taq polymerase. MY09/MY11 PCR was carried out in a reaction volume of 50 μl containing 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1 mM $MgCl_2$, 200 μM dNTPs, 1 μM each primer and 0.05 U/μl Taq polymerase. GP5$^+$/GP6$^+$ PCR was carried out in a reaction volume of 50 μl containing 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 3.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM each primer and 0.05 U/μl Taq polymerase. Amplification for ß-globin and MY09/MY11 consisted of an initial denaturation stage at 95˚C for 10 min followed by 40 cycles of 2 min at 95˚C, 2 min at 57˚C and 2 min at 72˚C, with a final extension at 72˚C for 7 min. For GP5$^+$/GP6$^+$, an initial denaturation at 94˚C for 10 min followed by 40 cycles of 1 min at 94˚C, 2 min at 40˚C and 90 sec at 72˚C, with a final extension at 72˚C for 7 min, was used. After amplification, PCR products were separated by electrophoresis on 3% agarose gel stained by ethidium bromide.

HPV genotyping. DNA samples showing positivity for MY09/MY11 and/or GP5$^+$/GP6$^+$ were analyzed by the INNO-line probe assay (LiPA) HPV Genotyping v2 amp kit and the INNO-LiPA HPV Genotyping v2 kit (Innogenetics) according to the manufacturer's protocol in order to identify the specific HPV genotype infecting the sample. Briefly, the INNO-LiPA HPV Genotyping v2 amp kit is designed to amplify a part of the L1 region of the HPV genome using PCR amplification with biotinylated primers. Use of this kit is based on the reverse hybridization principle, where denaturated biotinylated amplicons are hybridized with specific oligonucleotide probes, which are immobilized as parallel lines on membrane strips. After hybridization and stringent washing, streptavidine-conjugated alkaline phosphatase is added and binds to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen yields a purple precipitate, and the results can be visually interpreted.

Results

PCR analysis and HPV genotyping. Of the 30 DNA samples extracted from paraffin-embedded breast cancers of different histotypes, 24 were positive for ß-globin amplification and were consequently considered suitable for HPV PCR analysis. One of the 24 DNA samples showed positivity for MY09/MY11 and GP5$^+$/GP6$^+$ amplification. HPV genotyping as above only showed the presence of generic HPV DNA, and none of a specific virus genotype (Fig. 1A).

Notably, the HPV-positive DNA sample was extracted from a rare breast cancer variant, papillary breast cancer. For this reason, we decided to collect and analyze a further cohort of 32 formalin-fixed paraffin-embedded samples of papillary breast cancer in order to verify whether HPV infection is significantly related to the pathogenesis of this specific type of tumor.

Of the 32 DNA samples extracted from the cohort of papillary breast cancer specimens, 28 were positive for ß-globin amplification and were consequently considered suitable for HPV PCR analysis. One of the 28 DNA samples exhibited positivity for MY09/MY11 and GP5$^+$/GP6$^+$ amplification. HPV genotyping as above revealed the presence of the specific high-risk HPV66 virus genotype (Fig. 1B).

To evaluate the reliability of MY09/MY11 and GP5$^+$/GP6$^+$ PCR, using the INNO-LiPA HPV Genotyping v2 kit we analyzed an additional ten breast tumors randomly selected from among the MY09/MY11 and GP5$^+$/GP6$^+$ PCR negative cohort. This yielded the absence of HPV DNA by reverse hybridization as well (data not shown).

To sum up, 2 of 52 (4%) breast cancer specimens showed the presence of HPV infection. Both of these samples were of papillary breast cancer.

Discussion

The relationship between viruses and mammary tumors is known from the induction of mammary cancer in mice with

listopathological data of analyzed breast cancer patients.

Patient	Age	Tumor	Grade	Estrogen receptor (%)	Progesterone receptor (%)	p53 expression (%)	Mib-1/Ki-67 expression (%)	c-erbB2 expression (%)
1/06	50	IDC	3	<5	<5	<5	25	
2/06	42	IDC	2	50	<5	5	20	100
3/06	57	IPC	1	60	20		<5	
4/06	71	ILC		60	25	Negative	30	
5/06	48	IDC 80% IntraDC 20%	1	70	10	Negative	<10	
6/06	50	IDC 80% IntraDC 20%	2;3	20	Negative	Negative	20	90
7/06	69	ILC	2	90	30	Negative	10	30
8/06	48	ILC 80% *in situ* LC 20%	1	90	20	Negative	12	
9/06	36	ILC	2	60	60		5	Weak
10/06	64	IPC	2	80	Negative		5	
11/06	45	ILC	2	90	90	10	15	Diffuse
12/06	44	IDC	2	80	5	10	10	Weak
13/06	44	IDC	2	50	80	1	30	Weak
14/06	52	ITLC	1	80	60	<5	5	Weak
15/06	55	IDC	3	100	100	5	30	Weak
16/06	58	IDC	2	100	<10	<5	<10	Weak
17/06	37	IDC	2	80	15	Negative	10	
18/06	57	IDC	3	Negative	Negative	80	30	Weak
19/06	50	IDC	3	80	Negative	Negative	40	Intense/diffuse
20/06	57	ITLB	1	100	100		10	Incomplete
21/06	57	IDC	3	90	80	10	30	Intense/diffuse
22/06	42	IDC	3	<5	<5	10	20	Weak
23/06	58	IDC	3	Negative	Negative	Negative	60	Weak
24/06	54	IDC	3	90	Negative	Negative	20	Weak
25/06	44	IDC	2	Negative	Negative		15	Intense/diffuse
26/06	65	IDC	2	80	80		<5	
27/06	50	IDC	1	100	80			Incomplete
28/06	60	IDC	3	90	50		40	Weak
29/06	52	IDC	1	100	10	<5	15	Weak
30/06	44	IDC 60% IntraDC 40%	2	80	80	<5	<10	Weak

IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; IPC, infiltrating papillary carcinoma; ITLC, infiltrating tubular-lobular carcinoma; IntraDC, intraductal carcinoma.

the mouse mammary tumor virus (MMTV) (26,27). However, the data obtained from studies investigating the presence of viral sequences in cancer biopsies and cell lines have been controversial. A human equivalent of MMTV has been described in breast tumors, as well as in breast cancer cell lines (28). These results have been questioned and negated by others (29).

Human viral carcinogenesis is well established through studies that have demonstrated the relationship between cervical dysplasia, cancer, and HPVs (30). The suspicion that HPVs may also play a role in human breast cancer is based on the immortalization of normal human breast cells by HPV types 16 and 18 (9). However, data on the presence of HPV DNA in human mammary tumor biopsies are controversial (10-24).

In this study, out of 52 cases of breast cancer only two were found to be HPV DNA-positive. Both tumors were breast papillary carcinomas and showed the presence of untypable HPVX DNA and high risk HPV66 DNA, respectively.

We routinely use the INNO-LiPA HPV Genotyping v2 kit for HPV DNA analysis of cervical cancers, and were able to verify the robustness of the technique. Using the kit, we also analyzed an additional ten breast tumors randomly selected among the MY09/MY11 and $GP5^+/GP6^+$ PCR negative cohort in order to evaluate the reliability of MY09/MY11 and $GP5^+/GP6^+$ PCR. This yielded the absence of HPV DNA by reverse hybridization as well.

Our results suggest that HPV infection is not significant in mammary tumorigenesis, with the exception of particular tumor histotypes, such as papillary cancer. It has been suggested that HPVs may infect the epithelium of the nipple and the areola, and could be identified by recognizable histological features, as in HPV infection at other sites (23). The pathogenic mechanism involves the transfer of HPV in a

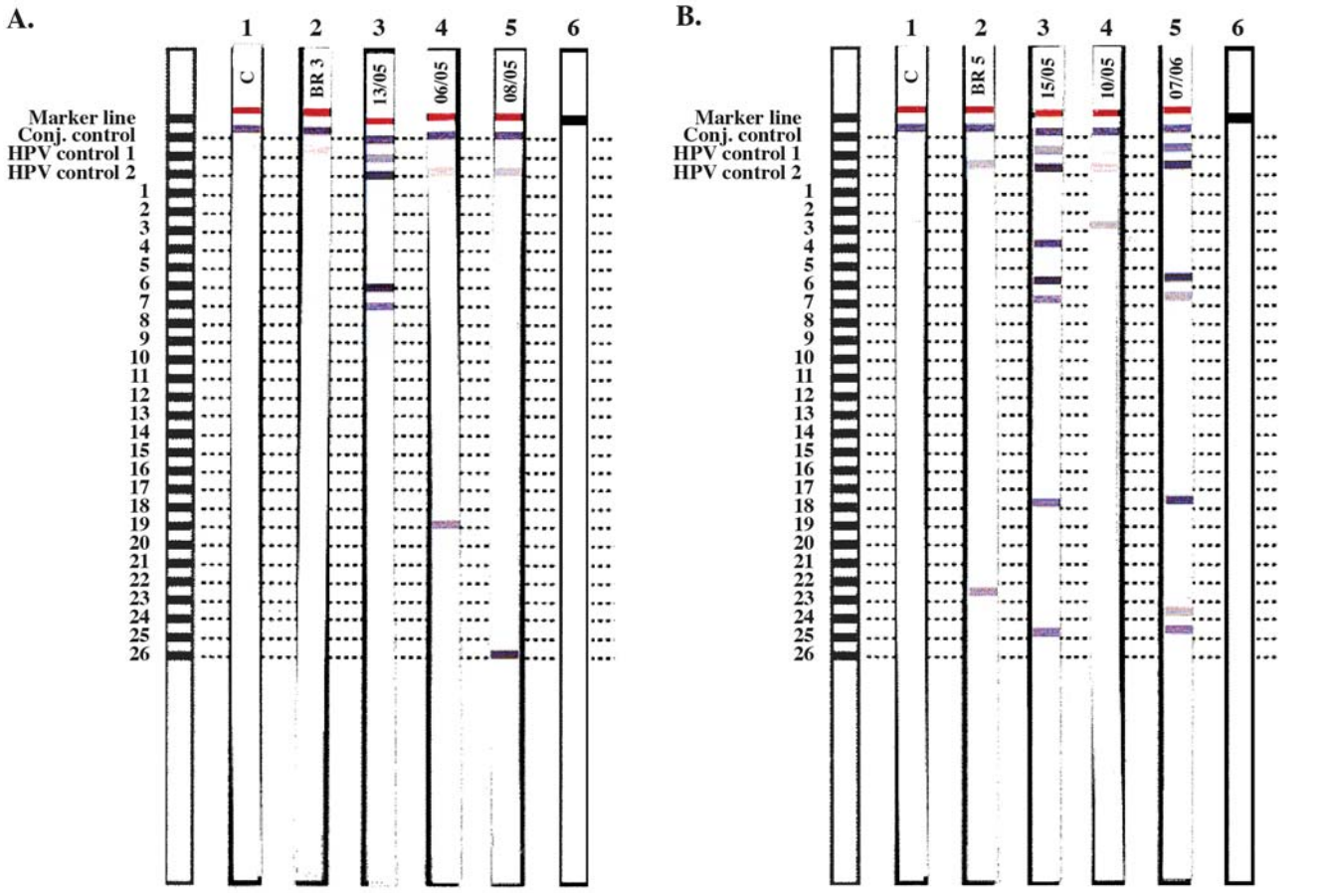

Figure 1. Reverse hybridization on INNO-LiPA strips. (A) Strip 1, negative control, no DNA; strip 2, HPV DNA- positive breast cancer (HPVX$^+$); strips 3-5, HPV-positive controls (cervical tumors). (B) Strip 1, negative control, no DNA; strip 2, HPV DNA-positive breast cancer (HPV66$^+$); strips 3-5, HPV-positive controls (cervical tumors).

retrograde fashion via the nipple, areola, lactiferous ducts and sinuses (23).

Our data appear to be in accord with findings that low risk HPV is rarely identified in benign squamous papillomas of the mammary nipple (31). Moreover, other researchers have failed to demonstrate the presence of HPV DNA in samples of breast carcinoma (18-22). Differences in the prevalence of HPV infection in tumors of the breast could be due to the particular population analyzed. For example, in Japanese-Chinese cohorts, a prevalence of the HPV33 genotype (12,13) has been demonstrated. This is in contrast to other countries, such as Australia, Austria and Brasil, where the presence of the HPV16 and HPV18 genotypes has primarily been found (11,15,17).

Acknowledgements

We thank Innogenetics for the INNO-LiPA HPV Genotyping v2 kit for breast cancer sample characterization.

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