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Analysis of the presence of cutaneous and mucosal papillomavirus types in ductal lavage fluid, milk and colostrum to evaluate its role in breast carcinogenesis

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Abstract Several independent studies have presented evidence for the involvement of human papillomaviruses (HPV) in the aetiology of human breast cancer, while others have reported the opposite findings. Here, we have analysed by a high sensitive multiplex PCR-based method the prevalence of alpha mucosal and beta cutaneous HPV DNA in 90 ductal lavages, colostrum and milk. Ten of the 70 DLs analyzed (14%) contained a single or multiple beta HPV types, while DNA from mucosal high-risk HPV types was detected in only one sample (1/70). A strong reduction of HPV positivity in DL fluids was observed in 45 specimens collected after removal of the superficial layers of the

nipple epidermis. All DLs were negative for the mucosal low-risk HPV types 6 and 11. Finally, HPV positivity was low in colostrum and milk. Our data show that DNA of alpha mucosa and beta cutaneous HPV types are rarely present in the breast fluids and suggest that a direct role of HPV in breast carcinogenesis is unlikely.

Keywords HPV · Breast cancer · Ductal lavage · Breast fluids

Introduction

The possible involvement of the epithelio-tropic human papillomavirus (HPV) in breast cancer has been proposed repeatedly in recent decades [1]. The HPV family comprises approximately 100 different types that have been sub-grouped in different genera according to their genomic DNA sequence [2]. In addition, the HPVs can be subdivided into mucosal or cutaneous, based on their tissue tropism. At least 15 different mucosal HPVs of genus alpha, namely 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, have been clearly linked to cervical cancer and classified as high-risk types [3]. Three additional HPV types of the same genus are classified as probable high-risk types, 26, 53 and 66, while several others, including HPV6 and 11, are considered low-risk types that are normally associated with benign cervical lesions [3]. Emerging lines of evidence indicate that also another group of HPVs that belongs to the genus beta may be involved in human carcinogenesis, i.e., non-melanoma skin cancer (NMSC) [4]. In vitro studies have shown that the main oncoproteins E6 and E7 from HPV16 are able to immortalise primary mammary epithelial cells, providing additional evidence for a possible role of this virus in breast carcinogenesis [5].

Massimiliano Cazzaniga, Debora Macis, Francesco Valenti and Mara Jo Miller collected and processed the breast lavages. Tarik Gheit performed the HPV analysis. Noureen Khan and Suminori Akiba collected the colostrum and milk specimens. Bakary S. Sylla, Andrea Decensi, Umberto Veronesi, Bernardo Bonanni, Chiara Casadio and Massimo Tommasino made the study design and prepared the manuscript.

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However, studies aiming to determine the presence of HPV DNAs in breast lesions by different polymerase-chain reaction (PCR)-based methods have produced contrasting results. Low or high prevalence of HPV DNA in breast cancer of women was found in several studies. HPV16 or HPV18 were preferentially found in European women, while other types, e.g., 33, were frequently found in Japanese and Chinese cohorts [6–16]. De Villiers et al. have recently shown that a broad spectrum of mucosal and cutaneous HPV types could be found in breast specimens using several PCR-based methods [16]. In the same study the presence of the low-risk HPV types 6 and 11 in breast tissues was also proved by *in situ* hybridization [16]. Additional studies have reported a high prevalence of HPV DNA in breast cancer of women with a history of cervical pre-malignant and malignant lesions [17–19], suggesting that HPV DNA may migrate through bloodstream from the original site of infection to the breast, or alternatively, may be transmitted by hand from the female perineum to the breast. In contrast to the studies cited above, no DNA from low-risk and high-risk HPV types was detected in several other independent studies using type-specific and/or consensus PCR primers in Paget's diseases, papillomas, papillary carcinomas and infiltrating ductal breast carcinomas [20–26]. In particular, Gopalkrishna et al. reported that when breast cancer cells were collected by aspiration with a fine needle to avoid any possible contamination of surrounding tissues, no HPV DNA was detected in the tumour tissue [22].

To further assess the possibility that mucosal and/or cutaneous HPV types play a role in breast carcinogenesis, we determined their prevalence in breast ductal lavage (DL) fluids of women at increased risk for breast cancer. We also aimed to determine their prevalence in colostrum and milk in order to verify the prevalence of HPV in physiological fluids.

Materials and methods

Collection of the specimens

Ductal lavage specimens and superficial layers of the nipple epidermis

One hundred fifteen ductal lavage specimens (DLs) were collected from September 2006 to July 2007 at the European Institute of Oncology (Milan, Italy) in a consecutive series of 90 women at increased risk for breast cancer, i.e., history or presence of contralateral ($n = 62$) or ipsilateral ($n = 13$) breast cancer, or unaffected women with a BRCA1/2 mutation or having >10% of risk of mutation according to the Berry–Parmigiani model ($n = 40$) [27]

(age ranging from 24 to 65, average +45.29). The nipple area was washed with a solution containing 96% ethanol (Neoxinal, Milan, Italy). After insertion of the catheter in the duct, the part was anaesthetised by injection of 2 ml 2% lidocaine solution. Ducts were washed with 10–12 ml of physiological solution. Two ml of DL fluid was mixed with 2 ml of fixative (PreservCyt, Cytoc, Boxborough, MA, USA) and sent to the International Agency for Research on Cancer (IARC) for HPV typing.

After the first 70 procedures, the collection of 45 DLs was preceded by removal of the nipple epidermis by tape stripping. This procedure was introduced to reduce the risk of contamination of DLs with HPV DNA presence on the skin. For this purpose, a plaster (Fixomull, BDF Beiersdorf AG, Hamburg, Germany) was attached to the skin and stripped with sterile forceps after approximately 1 min. After the tape stripping, collection of DL fluid was performed as described above. The tapes were transferred in sterile cap tubes and sent to IARC for the detection of HPV DNA.

The ductal lavages were performed approximately 1 month after the surgical intervention and before the beginning of any type of therapy. No clear presence of malignant cells was detected by cytological analysis in all ductal lavages. In only four specimens we observed atypical suspicious malignancy. Of these four women, two had ipsilateral cancer, one had contralateral cancer and one is at >10% risk of mutation. All women were informed of the aim of the program and gave their written consent. This study was approved by the ethical committees of the European Institute of Oncology and IARC.

Colostrum and milk

To broaden our analysis to other breast fluids, we have extended our study to include milk and colostrum. We have chosen an Asian population to evaluate the possibility that the HPV prevalence in breast fluids may be influenced by genetic features. Twenty-five breast milk and ten colostrum samples were collected at the Department of Epidemiology and Preventive Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan. This evaluation was done in average risk women in order to determine the possible presence of HPV in physiological mammary fluids. After extensive washing of the nipple area and the hand of lactating mother, colostrum or milk was collected by dropping the liquid directly into sterile tubes. Precautions were taken to avoid any contact between the skin and the sterile tubes. All specimens were sent to IARC for the detection of HPV DNA. The study was approved by the ethical committees of Kagoshima University and IARC.

DNA extraction

Ductal lavage

DNA extraction was performed by using the Qiagen BioRobot EZ1 with the EZ1 DNA tissue kit and EZ1 DNA tissue card or QIAamp® UltraSens™ Virus kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). To monitor the possible occurrence of cross-contamination between the different specimens during DNA extraction, tubes containing buffer only were also included.

Tape attached material

DNA purification was performed using the QIAamp DNA Micro Kit according to the instructions provided by the manufacturer (QIAGEN, Hilden, Germany).

Colostrum and milk

DNA was isolated using QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany). For preparation of DNA, 200 µl native colostrum or milk was used. Lysis, digestion with proteinase, precipitation and elution of membrane-bound DNA were carried out following the instructions of the manufacturer.

DNA quality control

The quality of the DNA extracted from all specimens described above was controlled by amplification of part of the β -globin gene with the primers previously described [28]. Only a minority of specimens resulted β -globin negative, i.e., one sample for the first set of ductal lavages (1/70) and three samples after tape stripping (3/45), while all milk and colostrum specimens were β -globin positive.

Detection of HPV DNAs

Multiplex PCR and HPV typing

Detection of HPV DNA was performed using two recently-developed highly sensitive and specific assays [29, 30]. The two assays detected 19 mucosal high-risk and probable high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82) and 25 cutaneous HPV types from genus beta (HPV5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 47, 49, 75, 76, 80, 92, 93 and 96). The assay for the detection of mucosal HPV type was slightly modified with respect to the previous version [29], which resulted to be less sensitive than the GP5+/6+ reverse line blot assay in detecting HPV16. We have modified the PCR forward primer for HPV16 E7

(5'-TTATGAGCAATTAAATGACAGCTCAG-3'). The new version of the multiplex PCR assay showed a comparable sensitivity to the GP5+/6+ reverse line blot assay in detecting HPV16 DNA (data not shown).

Detection of HPV6 and 11 DNA

The presence of HPV6 and 11 DNA was determined by PCR using HPV type-specific primers (HPV6 E7 forward 5'-GTA TTA GAC CTG CAA CCT CCA GAC-3'; HPV6 E7 reverse 5'-GTG CGC AGA TGG GAC ACA CTA TG-3'; HPV11 E7 forward 5'-TAC TAG ACC TGC AGC CTC CTG AC-3'; HPV11 E7 reverse 5'-GAT GGG ACA CAC AAT ATT TAG TGT GC-3'). The size of the PCR products was 252 bp for HPV6 E7 and 244 bp for HPV11 E7.

Results

DNA from mucosal and cutaneous HPV types is detected in ductal lavage fluid

We initially tested the presence of HPV DNA in 70 DLs from women at increased risk for breast cancer (affected women, BRCA1/2 mutation carriers or >10% of risk of mutation according to the Berry–Parmigiani model [27]). Two different detection assays for 25 cutaneous beta and 19 mucosal high-risk alpha HPV types were used [29, 30].

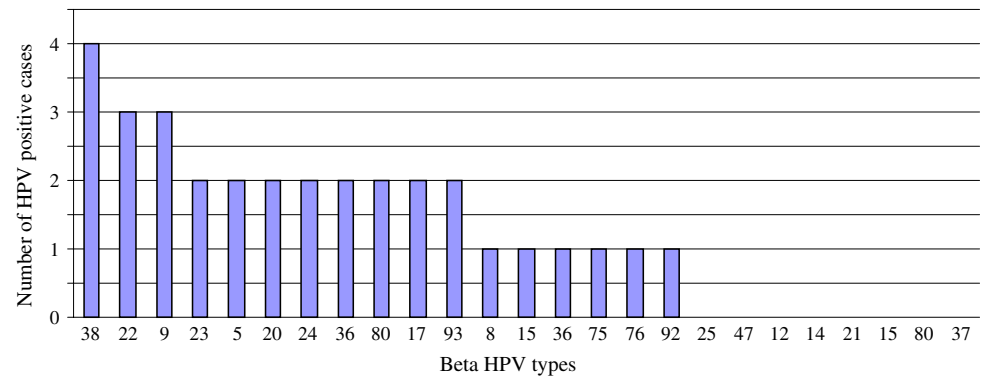
Only one specimen of the 70 (1.4%) tested positive for mucosal high-risk HPV DNA (type 51), while cutaneous beta HPV types were detected in ten ductal lavage specimens (10/70, 14%) (Table 1). Of ten cutaneous HPV-positive samples, seven were found to have more than one

Table 1 Single or multiple HPV DNAs detected in ten of the 70 DL fluids samples

Patient number	Alpha mucosal high-risk HPV types	Beta cutaneous HPV types
1	Negative	8
5	Negative	9, 38
9	Negative	92
13	Negative	20, 21, 22, 23, 36, 80
14	Negative	9, 17, 24, 38
16	Negative	75, 76, 93
26	51	5, 22
27	Negative	5, 9, 93
32	Negative	15, 17, 20, 22, 23, 24, 36, 38, 80
42	Negative	38

Patients not included in the Table tested negative for alpha and beta HPV types

Fig. 1 Detection frequency of beta HPV types in 70 DL fluids



HPV type. Altogether, 32 beta HPV DNAs were present in the ten breast specimens. HPV38 was the most frequently detected type, followed by types 9 and 22 (Fig. 1). HPV positivity was not preferentially associated with a group of women at specific risk, i.e., genetic predisposition or contralateral breast cancer (data not shown).

A recent study reported the presence of DNA from cutaneous and mucosal HPV types in areolar and cancer tissues from female patients with breast carcinoma [16]. In particular, the mucosal low-risk HPVs were found in breast tissue by PCR and in situ hybridisation. Therefore, we also searched for DNA of the low-risk mucosal HPV types 6 and 11, using E7 type-specific primers. No DNA from HPV6 and 11 was detected in the 70 DLs analysed.

Stripping of the superficial epidermis of the nipple strongly decreases HPV positivity in DLs

The collection of the DL fluids is performed using a catheter. Before insertion in the mammary ducts, the catheter may touch the nipple skin and contact any HPV flora present on the skin. Thus, the HPV DNA detection in the ten out of the 70 DL fluids positive for the beta HPV types may have been due to external contamination. Alternatively, the HPV positivity is associated with a real infection of the ducts. To discriminate between these two possibilities we introduced a modification in the procedure for the collection of the DL fluids. Forslund et al. reported a new method that consists in the removal of the superficial layers of the epidermis by tape stripping and found a strongly decreased HPV DNA prevalence in skin biopsies [31]. Based on these findings, the skin superficial layers of the nipple area were removed by tape before the collection of the DL fluids. We collected and analyzed 45 DLs for the presence of the alpha and beta HPV types. No DNA from the alpha HPVs were detected in DLs, while only two specimens contained a single infection with beta HPV types, i.e., 5 and 24. Thus, the HPV-positivity in DLs strongly decreased after stripping of the superficial layers of the nipple skin.

Beta HPV types are highly prevalent in the epidermis of the nipple

The very low prevalence of HPV DNA in DLs after tape stripping suggests that the nipple area is highly colonised by different HPV types. To confirm this hypothesis we extracted total DNA from the tape and determined the presence of the beta HPV types. A β -globin signal was obtained in 41 of the 45 samples (91%); of these 37 were positive for beta HPV DNA. The majority of the specimens contained multiple beta HPV types (Table 2). HPV DNA was also detected in two of the four β -globin negative samples (Table 2). HPV5, 12, 15, 24, 47 and 75 were the most prevalent types, being present in approximately 35% of the tape specimens (Fig. 2). Together, these data show that beta cutaneous HPV types are highly prevalent in the nipple skin area.

DNA of cutaneous and mucosal HPV types were seldom detected in colostrum or milk

To further investigate the presence of HPV DNA in mammary ducts, we analyzed ten and 25 samples of colostrum and milk, respectively. Only one sample (1/10, 10%) of colostrum contained the mucosal high-risk HPV16, while two milk samples resulted positive for the beta HPV type 5 or 23. In line with the data obtained in DL fluids, the analysis of the milk and colostrum indicates that HPV DNA is very rarely present in mammary ducts.

Discussion

The possible involvement of HPV in breast carcinogenesis is still under debate. Many studies have reported the presence of HPV DNA in breast tissues, while others did not confirm the findings [1]. Damini et al. found 25% of HPV positivity in 101 breast cancer tissues [14]; moreover, Kroupis et al. [6] showed a 16% rate of HPV positivity in more than 100 breast cancer tissue specimens, and they suggested a

Table 2 Single or multiple HPV DNA in each of the 45 tapes containing nipple epidermis

Patient number	β -globin	Beta cutaneous HPV types
1	+	12, 15, 17
2	–	5, 15, 36, 75
3	+	12, 15
4	+	5, 9, 12, 15, 17, 21, 23, 47
5	+	5, 12, 15, 19, 20, 24, 38, 47, 75
6	+	5, 47
7	+	–
8	+	5, 12, 15, 24
9	+	5, 8, 12, 23, 24, 47, 80
10	+	5, 15, 20, 23, 24, 75
11	+	14, 15, 20, 22, 24, 36
12	+	12, 22, 38, 47, 75
13	+	5, 12, 20, 24, 38, 75
14	+	15, 24, 47
15	–	–
16	+	8, 14, 20, 23, 24, 36, 38, 47, 75, 80
17	+	8, 19, 36, 80
18	+	5, 8, 12, 20, 23, 24, 38, 93
19	+	5, 14, 23, 24
20	+	15, 20
21	+	5, 12, 20, 21, 22, 23, 24, 47, 75, 80
22	+	15, 47
23	+	15, 23, 24
24	+	5, 12, 15, 17, 23, 25
25	+	5, 15, 17
26	+	5
27	+	12, 47
28	–	93
29	+	5, 22, 36
30	+	12, 19, 22, 36, 75, 92
31	–	–
32	+	5, 22, 24
33	+	12
34	+	19, 23
35	+	15, 24, 75
36	+	75
37	+	5, 12, 15, 23, 47, 75
38	+	8, 15, 17, 47
39	+	–
40	+	–
41	+	20, 22
42	+	9, 23, 47, 75
43	+	8, 20, 24, 47, 75
44	+	8, 47
45	+	–

correlation between infection and elevated tumour grading and proliferation index over a low ER+ expression. However, Lindel et al. [26] did not support these findings.

All of these data are derived from histological samples of breast cancer tissue; our study is the first to look for an eventual HPV infection in cytological samples. In fact, we have analysed the presence of the DNA from mucosal and cutaneous HPV types in the lavage fluid of mammary ducts. Cutaneous beta HPV types were found in 14% (10/70) of the breast specimens, while DNA from mucosal high-risk HPV types was detected in only one sample (i.e., HPV51) (1.4%). However, when the collection of DL fluids was preceded by the removal of the superficial layers of the nipple epidermis by tape stripping, the HPV positivity was dramatically reduced, dropping from a total number of HPV infections of 32 to 2. These findings suggest that the majority, if not all, of the HPV DNA detected in DL fluids may have been transported from the epidermis into the ducts by the catheter.

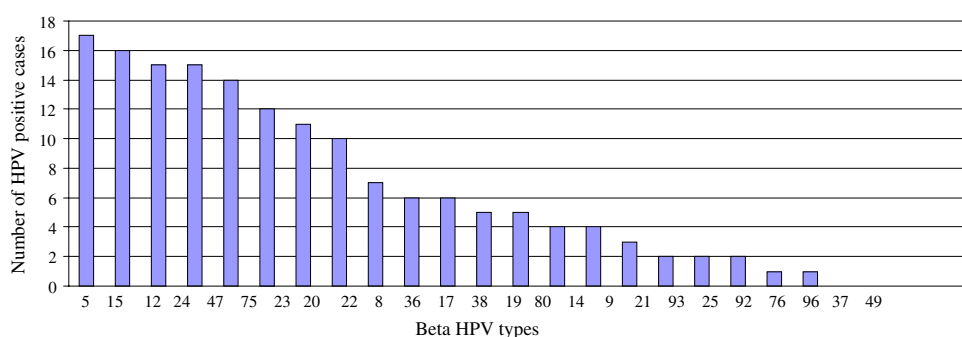
The collection of colostrum and milk samples did not involve any mechanical stress, and HPV positivity was extremely low in these specimens, i.e., three HPV DNAs in 35 samples ($\cong 8\%$).

Surprisingly, the spectrum of the HPV types detected in the 70 DL fluid collected without tape stripping differs from the one detected in the material extracted from the 45 tapes (Figs. 1 and 2). Different hypotheses can be envisaged to explain these findings. It is possible that the most frequently detected HPV types in the DL fluids (9, 22, 23 and 38) are present on the skin of the nipple at a higher copy number than the other beta HPVs. Therefore, they have a higher probability than the others to be transported by the catheter into or infect the ducts. Alternatively, the different HPV spectrum detected in DLs and tapes may be simply due to the fact that the two types of specimens were obtained from two distinct groups of women. However, since the women included in the two groups do not show any apparent difference, e.g., race and place of residence, and were randomly selected, the second hypothesis is less probable. In future studies, we intend to determine the copy number of the different beta HPV types present at the nipple epidermis by real-time PCR.

Our current data do not reveal whether the few HPV DNAs detected in the colostrum, milk and DLs collected after skin stripping correspond to real HPV infections. The detection of viral transcripts in cytological material will provide convincing proof for the presence or absence of an active HPV infection in the mammary ducts.

In addition to HPV, several studies have provided evidence for the involvement of other viruses in breast

Fig. 2 Detection frequency of beta HPV types in 45 samples of the nipple epidermis removed by tape



carcinogenesis, e.g., cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus (HHV)-8 and mouse mammary tumour virus (MMTV)-like virus [1]. The evaluation of the presence of these viruses in DL fluids may provide important insight into their possible role in breast cancer.

In conclusion, our findings show a very limited presence of HPV in ductal lavage fluid, colostrums and milk, and do not support a major role of HPV in breast carcinogenesis. Further methodological approaches are needed to improve our ability to evaluate the presence of HPV infection in cytological samples, but at present a direct role of HPV in breast carcinogenesis appears to be unlikely.

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