Human Papillomavirus and Breast Cancer: No Evidence of Association in a Spanish Set of Cases

Maria Vernet-Tomas\textsuperscript{1,2,3}, Marisa Mena\textsuperscript{4}, Laia Alemany\textsuperscript{4,5}, Ignacio Bravo\textsuperscript{4}, Silvia de Sanjose\textsuperscript{4,5}, Pau Nicolau\textsuperscript{1}, Alba Bergueiro\textsuperscript{1}, Josep Maria Corominas\textsuperscript{2,3,6}, Sergi Serrano\textsuperscript{3,6}, Ramon Carreras\textsuperscript{1,3} and Belen Lloveras\textsuperscript{6}

\textsuperscript{1}Obstetrics and Gynaecology Department, \textsuperscript{2}Breast Cancer Unit, and \textsuperscript{6}Pathology Department, Hospital del Mar, Barcelona, Spain;
\textsuperscript{3}Department of Paediatrics, Obstetrics and Gynaecology and Preventive Medicine, Autonomous University of Barcelona, Barcelona, Spain;
\textsuperscript{4}Unit of Infections and Cancer, Cancer Epidemiology and Research Program, IDIBELL, Catalan Institute of Oncology, Hospitalet de Llobregat, Spain;
\textsuperscript{5}CIBER Epidemiology and Public Health, Barcelona, Spain

Correspondence to: Maria Vernet-Tomas, Obstetrics and Gynaecology Department, Hospital del Mar, Passeig Maritim 25-29, 08003 Barcelona, Spain, e-mail: mvernet@hospitaldelmar.cat

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Abstract. Background/aim: Great controversy exists about the association between Human Papillomavirus (HPV) and breast tumours. The aim of this study was to explore the presence of HPV DNA in a large set of breast cancer cases. Materials and Methods: Techniques used followed the standards for an international retrospective survey of HPV-DNA genotyping, coordinated by our own group and the DDL Laboratories in Rijswijk (the Netherlands). Paraffin-embedded samples were used. SPF-10 broad-spectrum primers were applied, followed by deoxynucleic acid enzyme immunoassay and genotyping by reverse-line probe assay (LiPA25, version 1).

Results: A total of 78 samples were included in the study, two from benign conditions and 76 carcinomas, including different histological subtypes. HPV was not present in any of the specimens studied irrespective of histology, hormonal status and of stage of disease. Conclusion: Our data do not support the involvement of HPV in breast carcinogenesis as no evidence of its presence was found.
Breast cancer is the second most common malignancy considering both sexes and the first cause of cancer mortality in women in less developed regions, with 1.67 million new cases and 522,000 deaths estimated annually (1). Its worldwide prevalence is still on the rise (2). In Spain, breast cancer is the most prevalent malignancy in women (excluding non-melanoma cutaneous tumours) (1). Even if a global downturn in its incidence has been observed since 2000 in some regions of our country because of mammographic screening saturation (3), a worrisome increase of breast cancer diagnosis in women under 45 years has been reported in Spain since 2001, attributed to changes in reproductive patterns, lifestyle and diet (4).

Despite great efforts in research including the exploration of different aspects of the non-hereditary form of this carcinoma, little is really known about its aetiology.

The possibility that viruses may play a role in breast carcinogenesis has been investigated for several years. The three viruses most studied as oncogenic factors in any step of breast cancer development are the Epstein–Barr virus, Mouse Mammary Tumour virus and Human Papillomavirus (HPV) (5).

The hypothesis that HPV may induce breast carcinogenesis is of great interest considering that HPV has been detected in many epithelial neoplasms. Since 2007, two prophylactic vaccines have been available with demonstrated efficacy to protect against infection produced by the two most common high-risk genotypes in cervical cancer, HPV16 and 18 (6); therefore the demonstration of HPV in any type of cancer suggests that vaccines should be tested as a possible preventive option.
As shown in the review of Alibek et al. on the role of viruses in breast cancer (7), the literature on HPV presence in breast malignant tumours has reported controversial results, some of them denying any role of HPV in breast cancer as little or no presence of HPV DNA was observed in any breast tumour sample, while others reported the prevalence of HPV-DNA in breast cancer to be as high as 86%. Recent studies have found prevalence ranging from 18% to 50% (8-12). Some authors have linked HPV presence in breast tumours with specific clinical presentations, such as juvenile breast cancer (13), or with particular pathological subtypes, as metaplastic breast carcinoma (14). To the best of our knowledge, there are no previous studies on HPV and breast cancer in Spain.

The objective of our study was to investigate the prevalence of HPV-DNA in breast cancer cases diagnosed in our setting, including different histological types and different clinical presentations, using a well-validated protocol.

Materials and Methods

Ethics. This study had formal ethic approval by the Ethical Committee for Clinical and Epidemiological research of our research centre, the Institut Mar d'Investigacions Mèdiques, approval number 2010/3866/I. Specimens were received anonymously and allocated a unique identification number upon reception.

Study design and samples. Samples from women diagnosed between 2000 and 2011 at the Hospital del Mar, Barcelona (Spain) and whose first treatment was surgery were evaluated. Paraffin-embedded specimens obtained during
surgical treatment were selected consecutively until achieving a maximum number of specimens representing the most frequent histological types of breast cancer and clinical presentations, and then enriched with the most rare ones in order to have representation of all histological diagnoses.

Data were collected on age, date at diagnosis and pTNM. Different phenotypic features such as hormone receptor (HR) expression [whether oestrogen receptor (ER) or progesterone receptor (PR) were positive], p53 overexpression, Human Epidermal Growth Factor Receptor 2 (HER2Neu) overexpression and Ki67 index were collected. Following the protocols established by our Pathology Department at the Hospital del Mar, ER and PR were considered positive if any percentage of tumour cells in the specimen with any intensity was stained; p53 was considered positive if 10% or more tumour cells stained; HER2Neu was considered positive if membranous staining was intense by immunohistochemistry or gene amplification was confirmed by fluorescent in situ hybridization; finally, the Ki67 index was established using a semiquantitative scale calculating the percentage of cells with positivity.

The specimens were classified into molecular subtypes using immunohistochemistry surrogates (15). Luminal A tumours were those positive for ER or PR but with a Ki67 index of less than 15%; luminal B tumours were tumours with positive ER or PR but with a Ki67 index of 15% or more or positivity for HER2Neu; triple-negative tumours were those without HR and negative for HER2Neu in the immunohistochemical study; finally, HER2 tumours were those negative for HR and with HER2Neu overexpression or gene amplification.
Pathology and laboratory procedures. Pathology procedures were performed at Hospital del Mar and laboratory procedures at the Catalan Institute of Oncology as previously described (16). Briefly, four paraffin sections were obtained for each block. Paraffin blocks from control samples of non-HPV-related tissues processed at the same time of the breast cancer cases were also analysed in order to check for contamination. Blocks were processed under strict conditions to avoid DNA contamination. First and last sections were stained with haematoxylin and eosin (H&E) for histopathological evaluation, which included confirmation of breast cancer. The histological type indicated in the pathology report was recorded and no further review was undertaken.

A sample was determined to be adequate for HPV testing if breast carcinoma or the lesion of interest was observed in both H&E-stained sections of the study specimen. Intermediate sections were used for HPV-DNA detection and genotyping. These were first treated with 250 μl of freshly prepared proteinase K solution to extract DNA. Short polymerase chain reaction (PCR) fragment using biotin labelled SPF10 primers was performed using 10 μl of a 1:10 dilution of the crude DNA isolate in a final reaction volume of 50 μl. The amplified PCR products were tested using a probe hybridization step with a cocktail of conservative probes recognizing at least 54 mucosal HPV genotypes for the detection of HPV DNA with a DNA enzyme immunoassay (DEIA). Optical densities (OD450) were read on a microtitre plate reader and categorized as HPV-DNA-negative, -positive, or borderline. Borderline samples were run on the DEIA system again. The amplimers of the DEIA HPV-DNA-positive samples would have been subsequently analysed by reverse hybridization line probe assay (LiPA25) (version 1; Laboratory Biomedical Products, Rijswijk, the

In order to evaluate DNA quality, the samples were subjected to a PCR targeting the human tubulin gene (forward primer: TCCTCCACTGGTACACAGGC; reverse primer: CATGTGCTCTCAGCCTCGG), which generated a 65 bp amplicon, the same size as the SPF10 amplicon used for assessing the presence of HPV-DNA.

Results

Eighty-six samples corresponding to 80 women diagnosed at the Hospital del Mar, Barcelona (Spain) between 2000 and 2011 were obtained. At least one block was available for processing for 80 cases; of these, two had to be discarded due to insufficient quality of the DNA according to the internal control amplification with the tubulin primers. From the 78 cases finally included in the study, two corresponded to benign conditions (sclerosing adenosis and fibroadenoma) and 76 to breast carcinomas. One out of 76 tumours was bilateral and the other 75 were single tumours.

The median patient age was 61.5 (range=28-89) years. The age distribution, pathology, pT, pN and the immunohistochemical features of the processed specimens are summarized in Table I. There was a representation of patients of all ages and several pathological subtypes, some of them rare, invasive ductal carcinoma being the most common, now called invasive carcinoma of no specified type. Seven cases of Paget’s disease, a carcinoma type affecting nipple epidermis, were also included.
Of the 76 carcinomas, all had information about hormonal receptors, p53 expression and HER2Neu expression. All stages of disease were represented, from pre-invasive status (in situ carcinoma, pTis) to locally advanced tumour (pT4b). Regarding invasive carcinomas (63 samples), the molecular subtypes defined by immunohistochemistry surrogates were all represented: although the information about Ki67 index was not available in 33 samples, luminal B subtype was assumed if hormonal receptors and HER2Neu were positive by immunohistochemistry, and the triple-negative subtype was assumed if hormonal receptors and HER2Neu showed negative staining.

None of the samples analysed contained HPV-DNA by means of the technique applied in this study.

**Discussion**

Despite being the most frequent malignancy and the second cause of cancer death in European and North-American women (2), breast cancer aetiology is still unknown. Several risk factors have been described and mathematical tools are used to calculate personal risk in order to apply preventive measures. Even if great amounts of time, effort and resources are devoted to research, not much can be really done in the 21st century to prevent the development of breast cancer in women.

The possibility that some viruses could be linked to breast cancer aetiopathogenesis has important public health implications. In fact the role of viruses in some types of cancer has been clearly established. One of them is HPV, as some genotypes were recognized to be carcinogenic in humans in 1995 (17). If HPV were to be involved in breast cancer aetiology, vaccination
against high-risk carcinogenic HPV virus should be tested as a possibility for preventing breast cancer.

The first authors to report the presence of HPV in breast cancer tissue were Di Lonardo et al. (18). After them, many other authors have studied the presence of this virus in breast tumours, finding varying detection rates ranging from 0 to 82% (7). In a systematic review by Simoes et al., including 29 primary studies and 2211 samples, they considered that overall HPV prevalence was 23%, with higher prevalence in North America and Australia than in Europe; considering nine case–control studies, they concluded that HPV was significantly associated with breast cancer, with an odds ratio of 5.9 (95% confidence interval=3.36-10.67) (19).

How HPV could reach breast tissue is not known, but its presence has been described in breast milk, which indicates that this virus can infect breast tissue (20). Breasts have open ducts contacting the environment and they are frequently involved in sexual activity, therefore a hypothesis could be that infection occurs through these contacts. Sexual intercourse is proven to be the most frequent route of HPV transmission, and malignancies related to high-risk HPV are mostly found in organs involved in sexual contact (21). Some authors, however, suggested that HPV could reach breast tissue through haematic dissemination, but neither this hypothesis nor the former have been confirmed (22). The oncogenic effect of HPV on breast tissue could be via E6 and E7 oncogenes, as in ano-genital cancer (7).

A point that supports HPV being a carcinogen for breast tissue is that oestrogens act to increase its oncogenic effect. Oestrogens are considered an independent risk factor for development of a clearly HPV-related neoplasm,
cervical cancer, especially since a meta-analysis including 24 epidemiological studies published in 2007 linked contraceptive use with this disease (23). Later in-vitro and in-vivo mouse assays showed that oestrogens cooperate with E7 HPV oncogene, transforming normal epithelium to a neoplastic one (24,25). Therefore, a parallel between cervical and breast tumours could be assumed as oestrogen exposure and high oestrogen levels are well established risk factors for development of breast cancer (26). Other co-carcinogens helping oestrogen exposure to trigger breast cancer development are not well known and HPV could, as in cervical cancer, be a good candidate.

Like in our study, many other authors have been unable to find any trace of HPV-DNA in breast tumours (27-35). The existence of contradictory data has created great controversy and several factors explaining false-positive or false-negative results have been described in several reviews about this subject (5,7,19,36). Reasons for false-negative results include the possible destruction of HPV-DNA during sample processing and fixation when paraffin-embedded tissues are analysed (36), the use of primers detecting L1 genes that may be disrupted after HPV integration into the host genome (37), or a low viral load of HPV when high viral replication stops and integration occurs (37). Reasons for false-positive results could be due to contamination during processing, very frequent especially in nested PCR (5), or cross-reactivity with non-carcinogenic HPVs from the skin (5). Applying a DNA quality control with the tubulin test, we rejected the possibility that our negative results were due to insufficient DNA preservation: although we found two samples testing negative, the rest of the samples showed good DNA quality and no evidence of HPV positivity.
We included the most common as well as the less common and rare types of breast cancer to explore the possibility that only certain histological types might be HPV related. We also included seven cases of Paget’s disease and 13 ductal in situ carcinomas (representing early steps in carcinogenesis) that could theoretically be more likely to contain HPV-DNA, but none gave a positive result by this very sensitive PCR method.

All the processing, contamination control and techniques used for this study followed the standards set for an international retrospective survey of HPV-DNA genotype distribution in HPV-related cancer worldwide designed and coordinated by the Catalan Institute of Oncology in Barcelona, Spain, and the DDL Laboratories in Rijswijk, the Netherlands (16). Moreover, the technique used in our research is the most sensitive PCR technique designed for HPV-DNA detection in tissues fixed in formalin and embedded in paraffin. The amplification of short DNA targets in HPV L1 gene (65 bp) ensures good results in most cases that may have poorly preserved DNA due to formalin fixation. Many studies in cervical and other genital cancer have demonstrated its suitability for this type of specimen (38). However, its high sensitivity can increase the risk of contamination between samples. In our study, strict practices to avoid contamination were undertaken and no positive samples were detected among negative controls.

Some researchers of our group have used the same technique to study HPV prevalence and distribution in cervical cancer cases from Spain (39), finding an adjusted prevalence of 89.1%. This result shows great contrast with the present study where no evidence of HPV presence was found but at the same time demonstrating the robustness and sensitivity of the technique used.
herein to detect HPV in different types of tumours. In the cervical cancer study, the authors found that diagnosis of cervical cancer in women over the age of 50 years was significantly associated with the risk of HPV negativity. As the mean age of the breast cancer cases included in the present study was 61.5 years, one could partially attribute negative results in our set of cases to age.

However, considering that we included 17 samples from women aged 50 years or less, which also tested negative for HPV, age likely does not explain the HPV negativity.

We conclude that after studying a large number of breast carcinomas of different histological types and clinical presentations using a highly sensitive PCR technique for paraffin-embedded tissues, we did not find evidence of any association between HPV and breast cancer.

Conflict of Interest

The Authors declare no conflicts of interest.

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References


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Table I. Age distribution, pathology type, pT, pN and molecular features by immunohistochemical surrogates of the processed samples.

<table>
<thead>
<tr>
<th>Features</th>
<th>Description</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age distribution, years</td>
<td>&lt;50</td>
<td>17 (21.8)</td>
</tr>
<tr>
<td></td>
<td>51-69</td>
<td>29 (37.2)</td>
</tr>
<tr>
<td></td>
<td>&gt;70</td>
<td>32 (41.0)</td>
</tr>
<tr>
<td>Pathological type</td>
<td>Carcinoma NST*</td>
<td>29 (37.2)</td>
</tr>
<tr>
<td></td>
<td>Invasive lobular</td>
<td>15 (19.3)</td>
</tr>
<tr>
<td></td>
<td>Mucinous carcinoma</td>
<td>9 (11.6)</td>
</tr>
<tr>
<td></td>
<td>Ductal carcinoma in situ</td>
<td>7 (8.9)</td>
</tr>
<tr>
<td></td>
<td>Paget disease of the nipple</td>
<td>7 (8.9)</td>
</tr>
<tr>
<td></td>
<td>Carcinoma with apocrine differentiation</td>
<td>7 (8.9)</td>
</tr>
<tr>
<td></td>
<td>Solid carcinoma</td>
<td>4 (5.2)</td>
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<tr>
<td></td>
<td>Mixed lobular carcinoma</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td></td>
<td>Carcinoma with signet ring cell differentiation</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td></td>
<td>Fibroadenoma</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td></td>
<td>Sclerosing adenosis</td>
<td>1 (1.3)</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pT</td>
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<td>13 (17.1)</td>
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<tr>
<td></td>
<td>pT1</td>
<td>24 (31.6)</td>
</tr>
<tr>
<td></td>
<td>pT2</td>
<td>26 (34.2)</td>
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<tr>
<td></td>
<td>pT3</td>
<td>10 (13.2)</td>
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<td></td>
<td>pT4</td>
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<tr>
<td>pN</td>
<td>pN0</td>
<td>45 (59.2)</td>
</tr>
<tr>
<td></td>
<td>pN1</td>
<td>20 (26.3)</td>
</tr>
<tr>
<td></td>
<td>pN2</td>
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<tr>
<td></td>
<td>pN3</td>
<td>7 (9.3)</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>2 (2.6)</td>
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<tr>
<td>Hormonal receptors*</td>
<td>Positive</td>
<td>40 (52.6)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>36 (47.4)</td>
</tr>
<tr>
<td>P53 expression*</td>
<td>Positive</td>
<td>17 (22.4)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>59 (77.6)</td>
</tr>
<tr>
<td>Ki67 index, % of cells*</td>
<td>&lt;14</td>
<td>23 (30.3)</td>
</tr>
<tr>
<td></td>
<td>&gt;14</td>
<td>20 (26.3)</td>
</tr>
<tr>
<td></td>
<td>Not reported</td>
<td>33 (43.4)</td>
</tr>
<tr>
<td>HER2Neu*</td>
<td>Positive</td>
<td>29 (38.2)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>47 (61.8)</td>
</tr>
<tr>
<td>Immunophenotypes of invasive tumors (n=63)</td>
<td>Luminal A</td>
<td>18 (28.6)</td>
</tr>
<tr>
<td></td>
<td>Luminal B</td>
<td>12 (19.0)</td>
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<tr>
<td></td>
<td>HER2Neu</td>
<td>8 (12.7)</td>
</tr>
<tr>
<td></td>
<td>Triple-negative</td>
<td>16 (25.4)</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
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</tr>
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</table>

HER2, Human Epidermal Growth Factor Receptor 2; NST, non-specified type; n, number of cases; * includes invasive and intraductal carcinoma.