Evaluation of E6 and E7 mRNA expression in HPV DNA positive breast cancer

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Summary

Several studies have suggested a possible role for HPV in the pathogenesis of the breast cancer. We investigated the presence of the HPV DNA in breast cancers and non malignant disease breast tissues by the use of a standard HPV detection method (INNO-Lipa HPV), in order to detect HPV DNA in metastatic nodes, to investigate a possible cervical HPV co-infection, and to evaluate the E6/E7 mRNA expression in HPV DNA positive breast cancer tissues. The rate of HPV infection was significantly higher in the cancer group than in controls (9/31 vs 0/12, p = 0.04). One out of eight metastatic axillary nodes was positive for HPV infection; 2/3 of the positive HPV breast cancer patients were co-infected at the cervical site. The role of the virus in breast oncogenesis is still unclear, since our analysis failed in demonstrating the expression of viral E6 and E7 in positive HPV positive breast tumor tissues.

Key words: Breast cancer; E6; E7; HPV; HPV 16.

Introduction

According with the Italian Network of Cancer Registries (AIRTUM), from 1998 to 2002 breast cancer was the most frequent cancer, representing 24.9% of all the cancer diagnoses in the AIRTUM area; mortality was 17.1% of all cancer deaths among females [1].

Although many risk factors have been established for this disease (e.g. age, family history or mutations of the BRCA1/2 genes), the etiology of the majority of breast cancers is unknown.

Over the last few years several studies have reported the detection of HPV within breast cancer tissues, suggesting a possible role for HPV in the pathogenesis of this tumor.

HPVs are classified in a "low-risk group" which generate benign lesions, and an oncogenic or "high-risk group (e.g. HPV-16, HPV-18) which is recognized as an etiologic agent of cervical and anal cancer [2, 3].

Papillomaviruses are a group of small non-enveloped DNA tumor viruses: to date, more than one hundred human and animal papillomavirus genotypes (types) have been completely sequenced. Viral gene expression leads to the expression of six nonstructural viral regulatory proteins (E1, E2, E4, E5, E6 and E7) and two structural viral capsid proteins (L1 and L2). E5, E6 and E7 are the viral oncogenes and their expression induces cell immortalization and transformation. In particular, E6 and E7 are two viral oncoproteins that inactivate, respectively p53 and Rb, two cellular tumor suppressor proteins [2].

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The correlation between high-risk HPV infections and cervical and anal carcinomas is widely accepted, but the correlation between HPV and malignancies of other tissues, including the breast, is still unclear.

Although the rate of HPV infection in breast cancer has been reported up to 86% [4], several authors failed in detecting the virus, resulting in an ample range of positivity among different studies [5-19].

Because of the contradicting results of the past studies, we conducted a search for HPV DNA in 31 breast cancers and 12 non malignant disease breast tissues through a gold standard detection method routinely employed for HPV detection and genotyping at the cervix; secondary aims of the present study were: a) to detect the HPV DNA in metastatic nodes, b) to investigate a possible cervical HPV co-infection in HPV positive breast cancer patients through DNA extraction and genotyping, and c) to evaluate E6 and E7 mRNA expression in HPVs DNA positive breast cancer tissues.

Material and Methods

Thirty-one women with an histological diagnosis of primary breast cancer were enrolled from 1994 to 2002. Mean age of the selected patients was 57 years (range 35-78); 29 were ductal carcinomas and two were lobular carcinomas.

Ten patients with diagnoses of breast fibroadenoma and two with diagnoses of breast papilloma were selected as a control group (mean age 27 years, range: 25-35).

Exclusion criteria were: past history of breast and cervical cancer and neo-adjuvant treatments. All molecular diagnostic investigations were performed on paraffin-embedded surgical

Table 1. — *Clinical and pathological features of breast cancer patients and controls*.

Patient	Histology	TNM	Breast HPV genotype	Cervical HPV genotype	Nodal HPV genotype	HPV mRNA expression
Cancer Group						
1	Ductal carcinoma	pT2N1	Negative		Negative	
2	Ductal carcinoma	pT1aN0	6	6, 11		
3	Ductal carcinoma	pT2N1	16	16	16	Negative
4	Ductal carcinoma	pT1cN0	Negative			
5	Ductal carcinoma	pT2N0	6, 51	6		
6	Ductal carcinoma	pT1N0	Negative			
7	Ductal carcinoma	pT1N0	Negative			
8	Ductal carcinoma	pT1cN0	16	16		Negative
9	Ductal carcinoma	pT2N0	Negative			
10	Ductal carcinoma	pT2N1	Negative		Negative	
11	Ductal carcinoma	pT1cN1	16	Negative	Negative	Negative
12	Ductal carcinoma	pT1cN0	51	51		
13	Ductal carcinoma	pT1cN0	18	16, 18		Negative
14	Ductal carcinoma	pT1cN1	Negative		Negative	
15	Ductal carcinoma	pT1cN1	Negative		Negative	
16	Ductal carcinoma	pT1cN0	Negative			
17	Ductal carcinoma	pT1cN0	16, 31	Negative		Negative
18	Ductal carcinoma	pT3N0	Negative			
19	Ductal carcinoma	pT1cN0	Negative			
20	Lobular carcinoma	pT2N0	Negative			
21	Ductal carcinoma	pT2N1	Negative		Negative	
22	Ductal carcinoma	pT2N1	Negative		Negative	
23	Ductal carcinoma	pT2N0	Negative			
24	Ductal carcinoma	pT2N0	56	Negative		
25	Ductal carcinoma	pT1N0	Negative			
26	Lobular carcinoma	pT2N0	Negative			
27	Ductal carcinoma	pT1aN0	Negative			
28	Ductal carcinoma	pT1cN0	Negative			
29	Ductal carcinoma	pT2 N0	Negative			
30	Ductal carcinoma	pT1bN0	Negative			
31	Ductal carcinoma	pT1cN0	Negative			
Control Group						
32	Fibroadenoma		Negative			
33	Fibroadenoma		Negative			
34	Fibroadenoma		Negative			
35	Fibroadenoma		Negative			
36	Papilloma		Negative			
37	Papilloma		Negative			
38	Fibroadenoma		Negative			
39	Fibroadenoma		Negative			
40	Fibroadenoma		Negative			
41	Fibroadenoma		Negative			
42	Fibroadenoma		Negative			
43	Fibroadenoma		Negative			

specimens; a signed consent for surgical treatment, cervical sampling and evaluation of the data was obtained from all selected patients. Clinical-pathological features of the study populations are shown in Table 1.

Cancer patients were treated with mastectomy or quadrantectomy plus lymphadenectomy according to the stage of the disease. Sentinel node biopsy was not performed since it was not a standard procedure in our Department at the time of this study. Control group patients were treated with lumpectomy.

All investigations for HPV genotyping were performed after DNA extraction from paraffin-embedded surgical specimens (cancers, fibroadenomas, papillomas and metastatic nodes) or from cytological cervical samples through the QiAamp DNA extraction kit (Qiagen) according to the manufacturer's protocol.

The detection of HPV was carried out through the INNO-Lipa HPV Genotyping kit, which can detect 28 different HPVs by DNA amplification followed by reverse hybridization of the SPF10 fragment (Innogenetics).

Stored frozen cancer tissues, positive for HPV 16, 18 and 31 DNA, were further investigated after RNA extraction for E6 and E7 mRNA expression by the NASBA assay Pre-Tect HPV-Proofer (Norchip), when the kit became available.

Briefly, NASBA is based on isothermal mRNA amplification, accomplished by the simultaneous enzymatic activity of avian myeloblastosis virus (AMV) reverse transcriptase, T7 RNA polymerase and RNase H. For detection primers and molecular beacon (MB) probes directed against E6/E7 mRNA for HPV types 16, 18, 31, 33, 45, 52 and 58 were employed. The final

concentration of MBs used in the reaction was 2.5 mM. NASBA amplification was carried out in a volume of 20 µl at 41°C for 2.5 h. A 5 µl volume of nucleic acids, diluted five times after the extraction was included in the reaction. The expression of U1A mRNA was evaluated as a performance control.

Statistical analysis was performed to compare the rate of HPV infection between cancers and controls: differences between groups were analyzed using the Fisher's exact test (MedCalc Software 11.3).

Results

HPV DNA was detected in nine out of 31 breast cancer patients (29%), and among these HPV 16 was the most frequent type detected (44%), followed by HPV 6 (22.2%). All controls resulted negative for HPV infection (Fisher's exact test $p = 0.04$). Table 1 summarizes the results of the study.

Patients who tested positive at the breast cancer site successively underwent cervical sampling to investigate a possible HPV co-infection at the genital site; six patients resulted to be (66%) co-infected. All these patients that tested positive for HPV at the genital site shared the same type (or at least one type) at both the breast and cervical sites.

Patients who tested positive for HPV at the cervix were successively referred for clinical gynecological evaluation and follow-up.

Eight patients in the cancer group had metastatic axillary nodes; among these just one patient (16%) tested positive for HPV infection, and the others resulted negative. Interestingly, this patient tested positive for HPV type 16 at the breast, node and cervical site.

After extraction of the RNA from stored frozen cancer tissues, we conducted a search for the expression of the E6 and E7 mRNAs in five patients who tested positive for HPV type 16, 18 or 31 at the breast site. Although human mRNA expression was positively validated by the housekeeping expression, the analysis failed in detecting the viral mRNA expressions in all the five samples.

Discussion

The relationship between HPV and breast cancer was first reported in 1992 [6-19]. Since then several authors investigated the presence of the virus in breast cancers through different techniques, resulting in an ample range of positivity among studies, ranging from 0 to 86.2% [4-19].

A literature review of previous studies reveals a high heterogeneity among HPV detection techniques, primer sets employed, tissues investigated (e.g. paraffin-embedded tissues, frozen tissues), population investigated and selection of controls. In our study, we investigated patients through a genotyping kit, which is routinely employed as a gold standard method for cervical HPV detection.

According to our results, the rate of HPV infection within breast cancer patients was significant if compared with controls ($p = 0.04$); moreover our detection rate was similar to what was previously reported by Khan and colleagues with the same detection method [20].

The possible role of HPV virus in carcinogenesis is indeed not clear: presence of the virus within the tumor could be coincidental, otherwise it might play a role causing chromosomal instability, inactivation of p53, activation of oncogenes or inactivation of tumor suppressors by insertional mutagenesis caused by integration of the virus within the host genome.

Key points in HPV-related cancers are integration of the virus in the host genome and expression of viral oncoproteins. Integration represents a consequence of viral infection and is detected in almost 90% of cervical carcinomas. The mechanism of integration is not completely understood, although there is a clear predilection for chromosomal common fragile sites due to their accessibility for insertion of foreign DNA. Indeed integration seems to be a direct consequence of chromosomal instability and an important molecular event in the progression of pre-neoplastic cervical lesions [21].

To clarify the viral status (integrated/episomal), Khan investigated the E2/E6 ratio and detected an integrated virus in the vast majority of the HPV-positive breast cancer tissues. However a mixed/episomal form was detected in the cervical cancer tissues (positive controls) and in the surrounding normal breast epithelium of the HPV positive tissues [20].

The authors moreover investigated the HPV viral load in positive breast tissues comparing results with HPV-positive cervical cancer tissues (positive controls), and highlighted that viral load of HPV-positive breast tissues was much lower than the one observed in cervical cancer tissues (geometric mean per 10^4 cells: 5.4 *vs* 130 480) [20].

We decided to investigate the viral oncoproteins, evaluating E6 and E7 mRNA expression in HPV DNA positive breast cancer tissues. According to our investigation, however, positive breast tumors did not express the viral E6/E7 transcripts, leaving unresolved the issues regarding the role of HPV in breast oncogenesis.

Two theories have been proposed to explain the possible mechanism through which the virus reaches the breast: one regards a mechanical transmission and the other one systemic spreading [4, 13]. De Villiers *et al*. detected the viruses in the nipple and the areola in patients with breast carcinoma, supporting the theory of a retrograde way in a retrograde fashion via the nipple through the breast ducts [4]. Widschwendter *et al*., hypothesized that the virus could reach the breast through the blood-stream after detecting the same HPV in nodes and breast cancers of cervical cancer patients [13].

In our study two-thirds of the patients who tested positive for HPV at the breast site resulted HPV positive at the cervical site, all of them shared at least one of the HPV types; however the mechanism of transmission remains unclear. To the best of our knowledge this is the first study aimed also to investigate the expression of HPV E6 and E7 mRNAs, the two major viral oncogenes in breast cancer tissues. In conclusion, these data indeed suggest that

further studies with the aim of investigating the presence of the virus in breast cancers should focus on the integration of the virus in host genomes and on the expression of viral mRNAs, in order to understand if the virus has an active role in breast carcinogenesis.

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