Brazilian Journal of Medical and Biological Research (2004) 37: 89-95
ISSN 0100-879X

Does the correlation between EBNA-1 and p63 expression in breast carcinomas provide a clue to tumorigenesis in Epstein-Barr virus-related breast malignancies?

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Abstract

Several investigators have identified Epstein-Barr virus (EBV) particles in breast carcinomas, a fact that supports a role for EBV in mammary tumorigenesis. The possible mechanism involved in this process is not clear. The present study was carried out in an attempt to determine whether there is a relationship between latent infection with EBV and p53 and p63 expression in breast carcinomas. Immunohistochemistry developed with 3.3-diaminobenzidine tetrahydrochloride was performed in 85 formalin-fixed paraffin-embedded breast carcinomas using anti-EBV EBNA-1, anti-p63, anti-p53, anti-estrogen receptor (ER) and anti-progesterone receptor (PR) antibodies. The cases were selected to represent each of the various histologic types: intraductal carcinoma (N = 12), grade I invasive ductal carcinoma (N = 15), grade II invasive ductal carcinoma (N = 15), grade III invasive ductal carcinoma (N = 15), tubular carcinoma (N = 8), lobular carcinoma (N = 10), and medullary carcinoma (N = 10). The ductal breast carcinomas were graded I, II and III based on the Scarff-Bloom and Richardson grading system modified by Elston and Ellis. One slide containing at least 1000 neoplastic cells was examined in each case. ER, PR, p63, p53 and EBNA-1 were positive in 60, 40, 11.8, 21.2 and 37.6% of carcinomas, respectively. There was a correlation between EBNA-1 and p63 expression ($P < 0.001$), but not between EBNA-1 and p53 ($P = 0.10$). These data suggest a possible role for p63 in the mammary tumorigenesis associated with Epstein-Barr virus infection.

Key words
- EBNA-1
- p63
- p53
- Breast
- Carcinoma
- Tumorigenesis

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Research supported by Fundação de Amparo ao Ensino, Pesquisa e Assistência, Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, SP, Brazil.

Publication supported by FAPESP.

Received January 9, 2003
Accepted September 30, 2003

Introduction

Epstein-Barr virus (EBV) is a ubiquitous DNA virus of the herpes family that causes infectious mononucleosis in humans. There is a strong relationship between latent EBV infection and the development of several malignant tumors including Burkitt's lymphoma, Hodgkin's disease, B cell lymphoma and nasopharyngeal carcinoma (1). Based on the association of the virus with these tumors, EBV has been classified as a group I

carcinogen by the WHO International Agency for Research on Cancer. Group I carcinogens are agents that definitely cause neoplasms in humans (2). There is also evidence that EBV could be related to other tumors, including gastric carcinoma, leiomyosarcoma, T cell lymphoma and lymphoepithelioma-like carcinoma of lungs, thymus, and salivary glands (3,4).

In recent years several investigators have identified latent EBV proteins in breast carcinomas, a fact that supports a role for EBV in mammary tumorigenesis (3,5,6). However, the possible mechanism involved in this process is not clear. One of the major tumorigenesis mechanisms of DNA viruses is to induce somatic mutations (7). It is well known that the most frequently mutated gene in breast carcinomas is the p53 tumor suppressor gene (8). When the p53 gene is mutated, its property of controlling the cell cycle is lost, leading to the development of neoplasms (9). In addition, a p53 gene homologue, named p63, has been identified (10,11). Despite their structural homology, these genes have different functional activities and the role of the p63 gene in carcinogenesis is still poorly understood (12).

The present study was carried out in an attempt to determine whether there is a relationship between latent EBV infection and p53 and p63 expression in breast carcinomas.

Material and Methods

Subjects

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local Ethics Committee. Eighty-five cases of breast carcinomas diagnosed from 1996 to 2001 were retrieved from the files of the Department of Pathology of the Ribeirão Preto School of Medicine. The criterion for selection was based on the histopathologic diagnosis. The cases were selected to represent each of the various histologic types, i.e., intraductal carcinoma (N = 12), grade I invasive ductal carcinoma (N = 15), grade II invasive ductal carcinoma (N = 15), grade III invasive ductal carcinoma (N = 15), tubular carcinoma (N = 8), lobular carcinoma (N = 10), and medullary carcinoma (N = 10). The ductal breast carcinomas were graded I, II and III based on the Scarff-Bloom and Richardson grading system modified by Elston and Ellis (13-15). None of patients had received any treatment before the biopsy procedure. For control purposes, 10 cases of normal breast tissue obtained from mammoplasty specimens and 10 cases of fibroadenoma were also selected at random. The clinical data were collected from the medical files and are summarized in Table 1. Standard clinical features were evaluated, including age, menstrual status, pathological grading, tumor size and lymph node metastasis. All patients were females.

Immunohistochemistry

All tissue samples had been routinely fixed in 4% neutral formalin and embedded in paraffin. Briefly, 3-µm thick sections were cut from paraffin blocks containing representative tumor samples. Paraffin sections were de-waxed in xylene, rehydrated through a series of graded alcohols, placed in 10 mM citrate buffer, and submitted to heat retrieval using a vapor lock for 40 min. After heating, the slides were allowed to cool to room temperature and briefly washed with Tris-buffered saline. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. Normal serum (Novocastra® Universal Quick kit, Benton Lane, UK) was used for 20 min in order to block nonspecific immunoassaying. Immunohistochemical staining was performed using a streptavidin-biotin peroxidase system (Novocastra®). The following primary antibodies were incubated for 2 h at room tem-

perature: p63 (1:200, clone 4A4; Santa Cruz®, Santa Cruz, CA, USA), p53 (1:100, clone DO7; Novocastra®), estrogen receptor (1:50, clone 6F11; Novocastra®), and progesterone receptor (1:50, clone 1A6; Novocastra®). Following washes in phosphate-buffered saline (PBS), biotinylated universal secondary antibody (Novocastra®) was applied for 15 min. The sections were incubated with the streptavidin-biotin complex reagent (Novocastra®) for 10 min and developed with 3.3-diaminobenzidine tetrahydrochloride in PBS, pH 7.5, containing 0.036% hydrogen peroxide for 5 min. Light Mayer's hematoxylin was applied as a counterstain. The slides were then dehydrated in a series of ethanols and mounted with Permount (Fischer®, Pittsburgh, PA, USA).

The rat monoclonal antibody clone 2B4 to EBV EBNA-1 was kindly provided by Dr. Friedrich Grässer (Abteilung Virologie, Institut fur Medizinische Mikrobilogie und Hygiene, Universitaatakliniken des Saarlandes, Hamburg, Germany). The protocol described by Bonnet et al. (3) was used for this antibody at 1:25 dilution.

Cases of invasive ductal carcinoma previously known to be positive for p53, anti-estrogen receptor or anti-progesterone receptor were used as positive controls. Normal skin was used as the positive control for p63, and nasopharyngeal carcinomas were used as a positive control for EBV EBNA-1. Negative controls for immunostaining were prepared by omission of the primary antibody. One slide containing at least 1000 neoplastic cells was examined in each case. Tumors were considered to be positive for EBNA-1, p63, p53, estrogen or progesterone receptors when their cells displayed a distinct brown nuclear staining. The slides were scanned and all cells were counted in a high-power field (40X). The cases were interpreted as p63, p53, estrogen or progesterone receptor positive if more than 10% of the neoplastic cells showed nuclear staining (16,17). The cases were interpreted as EBNA-1 positive if more than 1% of the neoplastic cells were stained.

Statistical analysis

Statistical analysis was performed using the Graph Pad Prism v.3 software (San Diego, CA, USA). Association between EBV EBNA-1 expression and other pathological variables was determined by the Fisher exact test (two groups) or by the chi-square test

Table 1. Epstein-Barr virus EBNA-1 expression and characteristics of patients and tumors.

Characteristic	No. of patients (%)	No. EBNA-1 positive (%)
Total	85 (100)	32 (37.6)
Age (years)		
<30	1 (1.1)	0 (–)
30-50	35 (41.1)	15 (42.8)
50-70	37 (43.5)	11 (29.7)
>70	12 (14.1)	6 (50)
Menopausal status		
Premenopausal	40 (47.0)	17 (42.5)
Postmenopausal	45 (52.9)	15 (33.3)
Pathological staging		
I	13 (15.2)	5 (38.4)
IIa	28 (32.9)	10 (35.7)
IIb	15 (17.6)	5 (33.3)
IIIa	11 (12.9)	2 (18.1)
IIIb	13 (15.2)	7 (53.8)
IV	5 (5.8)	3 (60)
Tumor size (cm)		
<2	22 (25.8)	7 (31.8)
2-5	49 (57.6)	19 (38.7)
>5	14 (16.4)	6 (42.8)
Lymph nodes		
Negative	47 (55.2)	16 (34)
1-3	23 (27.0)	10 (43.4)
>3	15 (17.6)	6 (40)
Histology[a]		
Ductal	45 (52.9)	23 (51.1)
S-B and R I[b]	15 (17.6)	6 (40)
S-B and R II[b]	15 (17.6)	4 (26.6)
S-B and R III[b]	15 (17.6)	13 (86.6)
Lobular	10 (11.7)	1 (10)
Medullary	10 (11.7)	5 (50)
Tubular	8 (9.4)	0 (–)
Intraductal	12 (14.1)	3 (25)

[a]All tumors were carcinoma. [b]Scarff-Bloom and Richardson (S-B and R) grading system modified by Elston and Ellis (13-15).

(three or more groups). All tests were 2-tailed, and a P value of <0.05 was considered to be significant.

Results

Estrogen and progesterone receptors were positive in 60 and 40% of the cases of carcinoma, respectively (Table 2). p53 was negative in all fibroadenomas and normal breast tissue and positive in 21.17% of the cases of carcinomas analyzed (Figure 1A). p53 was expressed in 26.6% of poorly differentiated carcinomas. In normal breast tissue and fibroadenomas p63 stained the nucleus of a single and continuous layer of cells surrounding the ductal and alveolar epithelium. p63 was totally negative in neoplastic cells of intraductal carcinomas, tubular carcinomas, lobular carcinomas, medullary carcinomas and grade I and II invasive ductal carcinomas. However, p63-positive cells were detected in 10 cases of poorly differentiated carcinomas (66.6% of grade III invasive ductal carcinomas, 11.76% of all breast carcinomas) (Figure 1B). Among these cases, only one co-expressed p53 (6.66% of grade III invasive ductal carcinomas, 1.17% of all breast carcinomas).

The results concerning EBNA-1 expression are summarized in Table 1. EBNA-1-positive cells were found in 37.6% of carcinomas analyzed. There was a strong correlation between EBV expression and the histological type of carcinoma. EBNA-1 was more

Table 2. Correlation of Epstein-Barr virus EBNA-1 with hormonal status and p53 and p63 expression in breast carcinomas.

Characteristic	No. of patients (%)	No. EBNA-1 positive (%)
Total	85 (100)	32 (37.6)
Estrogen receptor		
Positive	51 (60)	17 (33.3)
Negative	34 (40)	15 (44.1)
Progesterone receptor		
Positive	34 (40)	13 (38.2)
Negative	51 (60)	19 (37.2)
p53		
Positive	18 (21.1)	10 (55.5)
Negative	67 (78.8)	22 (32.8)
p63		
Positive	10 (11.7)	9 (90)
Negative	75 (88.2)	23 (30.6)

*P < 0.01 (Fisher test).

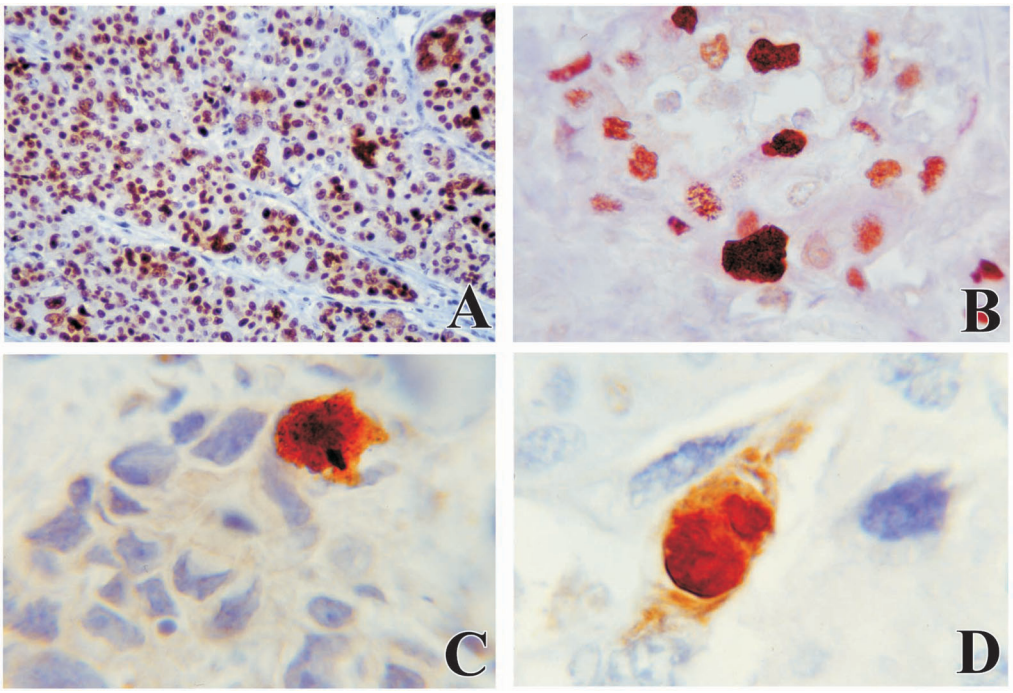

Figure 1. *A*, p53-positive cells in invasive ductal carcinoma (IHQ, p53, original magnification 20X). *B*, p63-positive cells in poorly differentiated ductal carcinoma (IHQ, p63, original magnification 40X). *C*, Neoplastic cell positive for EBNA-1 easily distinguishable from the adjacent non-stained cells (IHQ, EBNA-1, original magnification 40X). *D*, Nucleus of a neoplastic cell stained with EBNA-1 in a highly characteristic granular pattern (IHQ, EBNA-1, original magnification 100X). IHQ = immunohistochemistry.

prevalent among the ductal carcinomas (51.1%), especially poorly differentiated ones (86.6%). A quarter of intraductal carcinomas expressed EBNA-1; all of them grade III according to Van Nuys classification (18,19). The stained cells were easily distinguishable from the adjacent nonstained cells (Figure 1C). EBNA-1 stained only the nucleus of neoplastic cells in a highly characteristic granular pattern (Figure 1D). The stromal elements surrounding the neoplastic cells, including lymphocytes, were negative. All fibroadenomas and normal breast tissues were negative.

Discussion

The role of EBV in breast cancer is a subject of controversy, since some investigators failed to identify EBV particles in their samples (20-25). Other investigators, however, have found EBV latent proteins in up to 51% of the breast carcinomas analyzed (3,26,27). Furthermore, regions endemic for EBV have a high prevalence of breast carcinomas and, in these areas, positive samples showed higher loads of the EBV genome (5,6). These data suggest a relationship between latent EBV infection and the development of breast carcinomas.

Brazil is considered to have an intermediate endemic rate for EBV between the rates observed in Equatorial Africa and Northern Europe (28). In the present study, EBNA-1 was positive in 37.6% of carcinomas, being totally negative in the normal breast tissues and fibroadenomas. Unlike nasopharyngeal carcinoma used as a positive control, positivity for EBNA-1 was focal in breast carcinomas. This may reflect a minor degree of EBV affinity for breast tissue. Chu et al. (24) have also detected a focal positivity for EBV-related proteins in breast carcinomas. However, they have found no association with tumor size, grade or lymph node status. In the present study, there was a strong correlation between EBNA-1 positivity and poorly differentiated carcinomas ($P < 0.001$). EBNA-1 expression mainly in high-grade tumors may suggest that it can be an indicator of aggressiveness of breast carcinoma, a fact supporting a role of EBV in mammary tumorigenesis.

In our study there was no significant correlation between EBNA-1 and p53 expression ($P = 0.10$). p53 mutations were detected in some neoplasms related to EBV infection, such as Burkitt's lymphoma, B cell lymphomas, gastric carcinomas and head and neck tumors (29-31). On the other hand, nasopharyngeal carcinomas rarely have mutations in the p53 gene (2). These data suggest that Epstein-Barr virus may have several mechanisms of tumorigenesis whose importance varies according to the target tissue. According to this point of view, mutations induced in p53 could be effective in lymphomas, but not in nasopharyngeal or breast carcinomas.

We detected a strong correlation between EBNA-1 and p63 expression in breast carcinomas (Table 2; $P < 0.001$), suggesting that the expression of p63 may be required as part of the tumorigenic action of EBV. p63-positive cells were detected in 66.6% of grade III invasive ductal carcinomas. Among these cases only one co-expressed p53 (6.66% of grade III invasive ductal carcinomas). These findings are surprising because 26.6% of poorly differentiated tumors were positive for p53. These data suggest that p63, as also demonstrated for the other p53-homologue p73 (32,33), may act indirectly as an oncogene by inhibiting the expression of p53. The possibility that p63 simply allows infection of the tumor cells by EBV cannot be ruled out. According to this point of view, the virus might act as a co-factor in the initiation or promotion of cancer in genetically susceptible women.

Acknowledgments

We acknowledge Ms. Ana Maria Anselmi

Dorigan and Ms. Ana Maria Azevedo de Abreu for excellent technical assistance. We are grateful to Dr. Friedrich Grässer, Abteilung Virologie, Institut fur Medizinische Mikrobilogie und Hygiene, Universitätskliniken des Saarlandes, Hamburg, Germany, who kindly provided the rat monoclonal antibody clone 2B4 to EBV EBNA-1.

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