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ARTICLE

Is p63 reliable in detecting microinvasion in ductal carcinoma *in situ* of the breast?

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P63, a p53 homologue, is considered to be a marker of myoepithelial cells in breast tissue. This study was carried out to determine the sensitivity of p63 in detecting myoepithelial cells in DCIS and to compare the results obtained with smooth-muscle actin (1A4) in an attempt to verify the reliability of p63 as a possible marker of microinvasion in breast carcinoma. Fifteen DCIS of the breast were submitted to immunohistochemical analysis with anti-p63 and 1A4 antibodies and to a double immunolabeling study using p63 with 1A4. The double immunolabeling study showed that the same cells positive for p63 were also positive for 1A4. The three cases of DCIS with micro-invasion were negative for p63 and 1A4 in the foci of invasiveness. P63 staining was continuous in five of twelve cases of DCIS without microinvasion, being focal and discontinuous in 6 cases and completely negative in one case. Smooth-muscle actin staining was continuous in nine of twelve cases, including the five cases positive for p63. Smooth-muscle actin was focal and discontinuous in only two cases, which were also discontinuous for p63. The DCIS negative for p63 was also negative for 1A4. In conclusion, our results confirm the data of literature that p63 is a specific marker of myoepithelial cells in breast tissue. However, p63 is not as sensitive as 1A4 in staining myoepithelial cells and lack of p63 expression cannot be used as a reliable marker of invasiveness in ductal carcinoma in situ of the breast.
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Keywords: p63, smooth-muscle actin, ductal carcinoma *is situ*, microinvasion, breast

Introduction

Ductal carcinoma in situ (DCIS) of the breast with microinvasion can be defined as DCIS with infiltration of the periductal stroma by a few tumor cells, singly or in clusters.[3] The foci of microinvasion lack myoepithelial cells and this is the major histological criterion for the diagnosis of invasiveness in breast carcinomas. However, microinvasion is often associated with an altered, desmoplastic stroma (55%) or a dense lymphocyte infiltrate (39%).[19] In these cases, recognition of myoepithelial cells based only on routine hematoxylin-eosin sections may be very difficult. To highlight the myoepithelial cells, several immunohistochemical markers have been proposed, many of them directed against muscle-related antigens.[6,9,13] Barbareschi et al. verified that p63, a p53 homologue, is a selective marker of myoepithelial cells of human breast.[1] The reliability of this marker in detecting foci of microinvasion in DCIS of the breast, however, merits further investigation. This study was carried out to determine the sensitivity of p63 in detecting myoepithelial cells in DCIS and to compare the results obtained with smooth-muscle actin, worldwide used for this purpose, in an attempt to verify the reliability of p63 as a possible marker of microinvasion in breast carcinoma.

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Materials and Methods

Subject

This study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local Ethics Committee. Twelve cases of ductal carcinoma *in situ* of the breast diagnosed from 1996 to 2001 were

retrieved from the files of the Department of Pathology of the Ribeirão Preto School of Medicine. The criterion of selection was based on histopathologic diagnosis. None of these tumors had foci of micro-invasiveness. All tumors were classified according to the Van Nuys System.[5,20] There were also included three cases of high grade DCIS with easily identifiably areas of microinvasion. None of patients had received any treatment before the biopsy procedure. For control purposes, 10 cases of normal breast tissue obtained from mammoplasty specimens were also selected randomly. The clinical data were collected from the medical files. Conventional clinical features were evaluated, including age, menstrual status, pathological grading and tumor size. 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 minutes. 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 minutes. Normal serum (Novocastra® Universal Quick Kit, UK) was used for 20 minutes in order to block nonspecific immunoassaying. Immunohistochemical staining was performed using a streptavidin-biotin peroxidase system (Novocastra® Universal Quick Kit, UK). The primary antibodies p63 (1:200, clone 4A4, Santa Cruz®, USA) and smooth-muscle actin (1:250, clone 1A4, DAKO®, Denmark) were incubated for 2 hours at room temperature. Following washes in PBS, biotinylated universal secondary antibody (Novocastra® Universal Quick Kit, UK) was applied for 15 minutes. The sections were incubated with the streptavidin-biotin complex reagent (Novocastra® Universal Quick Kit, UK) for 10 minutes and developed with 3.3-diaminobenzidine tetrahydrochloride (DAB) in phosphate-buffered saline (pH 7.5) with 0.036% hydrogen peroxide for 5 minutes. Light Mayer's hematoxylin was applied as a counterstain. The slides were then dehydrated in a series of ethanols and mounted with Permount (Fischer®).

Normal skin and normal myocardium were used as positive controls for p63 and smooth-muscle actin, respectively. Negative controls for immunostaining were prepared by omission of the primary antibody. Tumors were scored as positive for p63 when their cells displayed a distinct brown nuclear staining, and positive for muscle actin when their cells displayed brown cytoplasmic staining.

The samples were also submitted to a double immunolabeling study according to the manufacturer protocol (DAKO EnVision™ Doublestain System, Denmark). For this purpose were used p63 (1:100, clone 4A4, Santa Cruz®, USA) with smooth-muscle actin (1:250, clone 1A4, DAKO®, Denmark). P63 (nuclear marker) was developed with DAB (brown) and the other antibodiy (cytoplasmic marker) was developed with fast red (red).

Results

Patient age ranged from 34 to 69 years, with a mean of 55 years. Seven patients were premenopausal and eight were postmenopausal. The tumors ranged in size from 0.7 to 5.5 cm, with a mean of 3.1 cm. Among the DCIS cases without microinvasion, seven patients had a Grade 3 tumor, four had a Grade 2 tumor, and one had a Grade 1 tumor according to Van Nuys classification.[5] The component *is situ* of the carcinomas with foci of microinvasion was grade 3 in all of three cases analyzed.

In normal breast tissue p63 stained the nucleus of a single and continuous layer of cells surrounding the ductal and alveolar epithelium. Smooth-muscle actin showed a similar staining pattern but involving the cytoplasm. The double immunolabeling study showed that both in normal and neoplastic tissue the same cells stained for p63 in the nucleus also were stained for smooth-muscle actin in their cytoplasm (*Figures 1A and 1B*).

In DCIS p63 staining was continuous in five of twelve cases analyzed (*Figure 1C*), being focal and discontinuous in 6 cases and completely negative in one case (*Figure 1D*), with positive external and internal controls. Smooth-muscle actin staining was continuous in nine of twelve cases (*Figure 1E*), including the five cases positive for p63. Smooth-muscle actin was focal and discontinuous in only two cases, which were also discontinuous for p63. The DCIS negative for p63 was also negative for smooth-muscle actin (*Figure 1F*), with positive external and internal controls. P63 and smooth-muscle actin were negative in the foci of micro-invasiveness in the three cases of DCIS with micro-invasion analyzed. All of these cases showed continuous positivity for smooth muscle actin in the areas *in situ*, while only one showed continuous positivity for p63.

Discussion

Breast carcinoma is one of the most common neoplasms in women and is a leading cause of cancer-related deaths worldwide.[14] Ductal carcinoma in situ (DCIS) of the breast is a heterogeneous group of lesions with diverse malignant potential. It is the most rapidly growing subgroup within the breast cancer family.[21] The recent increase in the diagnosis of DCIS is a result of current routine mammographic screening.[11] Of critical importance to the diagnosis of intraductal carcinoma is the integrity of the basement

membrane. When carcinoma is confined to the duct system, without violation of the basement membrane, axillary lymph node involvement is unlikely. However, lymph node metastases may be present in DCIS, probably due to foci of micro-invasiveness. So, the accuracy of diagnosis of invasiveness is important to predict the biological behavior of DCIS, although micro invasive breast carcinoma prognosis is only slightly worse than DCIS.[7]

High grade DCIS, especially of the comedo type, frequently has an associated lymphoid infiltrate with periduc-

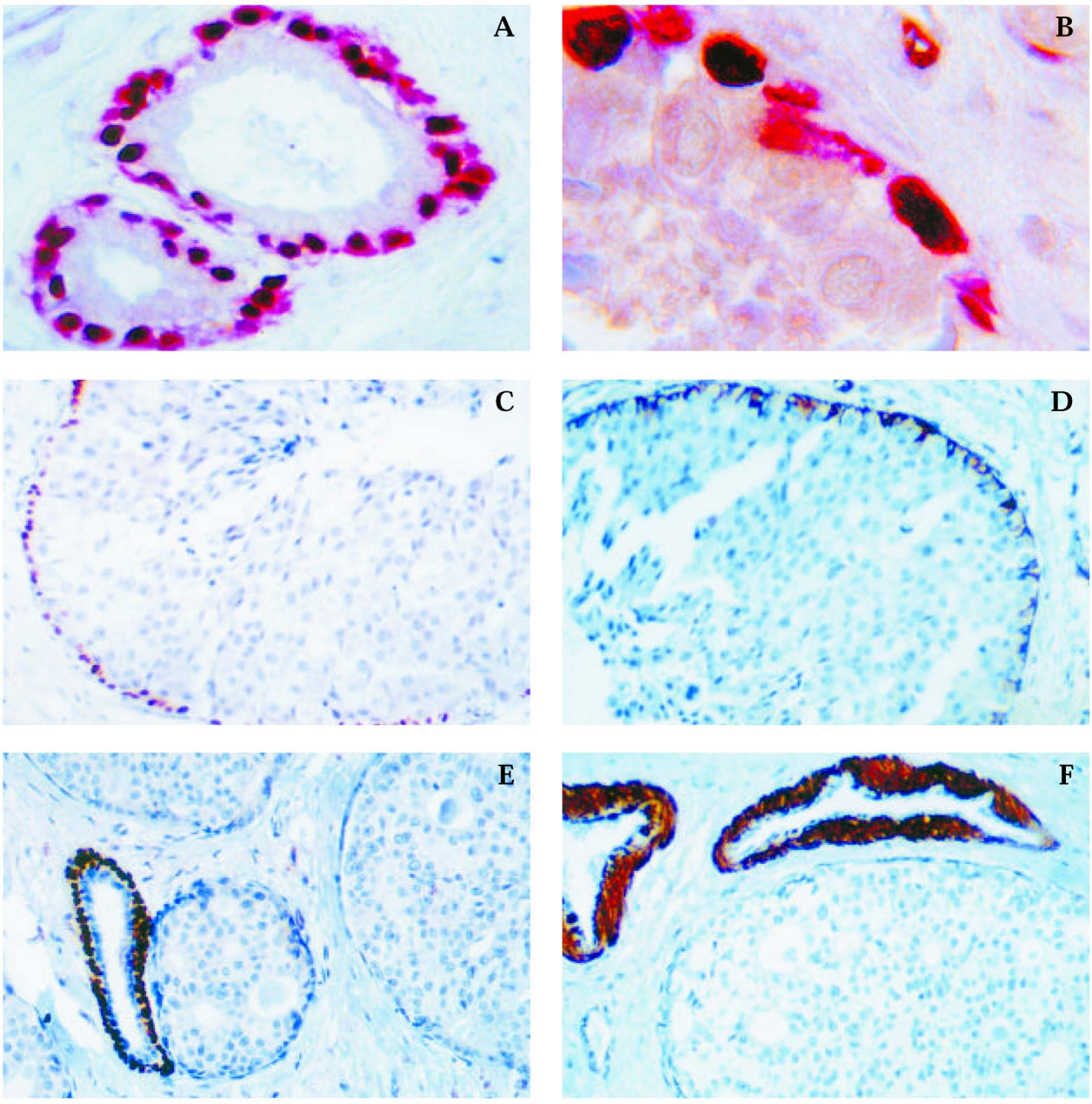

Figure 1. *(A) P63 (nucleus, brown) and smooth-muscle actin (cytoplasm, red) in normal breast tissue (double immunostaining, p63 with 1A4, original magnification X40), (B) P63 (nucleus, brown) and smooth-muscle actin (cytoplasm, red) in ductal carcinoma in situ of the breast (DCIS) (double immunostaining, p63 with 1A4, original magnification X100), (C) Continuous positivity for p63 in myoepithelial cells of DCIS (immunohistochemistry, p63, original magnification X20). (D) Negativity for p63 in myoepithelial cells of DCIS. Note a strongly stained normal mammary duct, acting as an internal control (immunohistochemistry, p63, original magnification X20). (E) Continuous positivity for smooth-muscle actin in myoepithelial cells of DCIS (immunohistochemistry, 1A4, original magnification X20). (F) Negativity for smooth-muscle actin in myoepithelial cells of DCIS Note the strongly stained wall of a blood vessel, acting as an internal control (immunohistochemistry, 1A4, original magnification X20).*

tal fibrosis and distortion and, particularly when 'cancerization' of lobules is present, it may mimic invasive carcinoma. Beyond these difficulties, the plane of section obtained from small ducts may simulate detached cell aggregates, mimicking invasion. In these cases, the immunohistochemical method is very helpful to detect myoepithelial cells, confirming or not suspected areas of invasion. However, the antibodies currently available to highlight myoepithelial cells, although precise in many cases, are not infallible, leading to equivocal diagnosis in some cases.[2] This may occur because these antibodies are mostly directed against the muscular antigens of myoepithelial cells. Barbareschi et al (2001) verified that p63, a p53 tumoral suppressor gene analogue, is a specific marker of myoepithelial cells in breast tissue.[1] The usefulness of p63 in detecting myoepithelial cells in breast tissue was confirmed by other authors.[15-17]

P63 maps to chromosome 3q27-29 and its product apparently is not a classical tumor-suppressor gene like p53.[10] The p63-deficient mice have defects of the apical ectodermal ridge essential to limb development and have truncated limbs. In addition, p63 null mice have no hair follicles, no teeth, and no mammary, lachrymal or salivary glands.[12, 23] These data suggest that p63 is necessary for normal development of epithelial organs and may be essential for the maintenance of a stem cell population in various epithelial tissues, being a marker of reserve cells. As a matter of fact, p63 stains the basal undifferentiated cells of some organs like skin and prostate, and may be considered to be an undifferentiated cell marker.[4,8,18]

In mammary epithelium there is a population of small stem cells located among the basal epithelium that are usually interpreted as being myoepithelial cells in sections stained with hematoxylin-eosin.[22] To verify whether p63 stained these cells or the myoepithelial cells we used double immunolabeling with p63 and smooth-muscle actin. The same cells that stained for p63 also stained for 1A4 confirming that these cells were indeed myoepithelial cells. Further studies will be necessary to explain why a stem cell marker is able to stain a type of well-differentiated and specialized cell.

As expected, in our work p63 was negative in the foci of microinvasion. However, we verified that p63 stained a continuous layer of myoepithelial cells in only five of twelve cases of DCIS analyzed without foci of micro-invasion, while smooth-muscle actin stained nine of them. Thus, lack of expression of p63 cannot be used as a parameter for invasiveness in DCIS. In conclusion, p63 is not as sensitive as smooth-muscle actin in staining myoepithelial cells and cannot be used as a reliable marker of invasiveness in ducal carcinoma in situ of the breast.

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