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Comparative Immunohistochemical Study of P63, SMA, CD10 and Calponin in Distinguishing *In Situ* from Invasive Breast Carcinoma

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Abstract

Background: Loss of the outer myoepithelial layer is the hallmark of invasive carcinoma, and demonstration of this loss can be documented by immunohistochemical techniques. The purpose of this study was to compare the specificity and sensitivity of four of the most commonly used-markers of myoepithelial cells: P63, SMA, CD10 and Calponin in distinguishing *in situ* from invasive breast carcinoma.

Material and methods: Immunostaining using antibodies against P63, SMA, CD10 and Calponin was performed on representative paraffin sections from 40 cases of breast masses examined at the Department of Pathology, Alexandria Faculty of Medicine, and diagnosed as ductal carcinoma *in situ* ± an invasive ductal or lobular carcinoma.

Results: Calponin yielded a slightly higher sensitivity than each of P63, CD10 and SMA (65% vs 54%, 19% and 17%, respectively). The results of both semiquantitative assessment and computerized image analysis of immunohistochemically-stained sections were statistically correlated, statistically p63 showed the highest specificity for myoepithelium and the least expression in non-myoepithelial layer of all antibodies tested. In contrast, SMA showed the least specificity and highest non-myoepithelial expression especially in stromal myofibroblasts and in vascular smooth muscle cells.

Conclusions: Calponin and P63 are more sensitive myoepithelial markers as compared to CD10 and SMA; with Calponin slightly more sensitive than P63. SMA should not be used alone as a myoepithelial marker due to its low specificity.

Keywords: Breast carcinoma; Myoepithelial cells; Cytokeratins; Mammary glands

Introduction

Myoepithelial cells (MEC) are contractile elements found in salivary, sweat, and mammary glands that show a combined smooth muscle and epithelial phenotype [1]. Normal breast glands and ducts are composed of 3 cell types that express different subsets of proteins: luminal, basal, and myoepithelial [1,2]. The luminal and basal cell types express different cytokeratins (CKs); myoepithelial cells (MECs) express basal cell-type CKs and other more specific markers, such as smooth muscle actin, calponin, and p63. An intact MEC layer is seen in both benign and *in situ* lesions, whereas loss of the MEC layer is considered the gold standard for the diagnosis of invasive cancer [2].

Carcinoma *in situ* (CIS) is defined as a proliferation of malignant epithelial cells confined by the basal lamina, whereas invasive carcinoma penetrates and grows beyond the basement membrane of the microanatomic structure in which it arises. Invasive ductal carcinoma, even the smallest, is treated differently from ductal carcinoma *in situ* (DCIS). Ruling out foci of invasion is most problematic in cases of extensive high-grade DCIS accompanied by prominent periductal stromal fibrosis and inflammation. Another diagnostic problem in which immunohistochemistry (IHC) can be of help is distinguishing ductal/lobular CIS-involving sclerosing adenosis or other complex sclerosing lesions from invasive carcinoma [3].

Earlier investigators approaching this problem stained for basal lamina components but found this could not reliably distinguish *in situ* from invasive tumors because some invasive carcinomas produce basement membrane components including laminin type IV collagen and type VII collagen [4].

Because MEC are not always readily identifiable on routine haematoxylin and eosin stained sections, many immunohistochemical methods have been used to highlight an intact MEC layer. Given the mixed epithelial and smooth muscle phenotype of MEC, and the need to distinguish the MEC layer from the epithelial cell layer, most of the markers used are directed against smooth muscle related antigens. Except in the rare cases of myoepithelial carcinoma usual ductal carcinoma cells are negative for MEC markers [3].

SMA is a sensitive marker of myoepithelial differentiation, but it is not specific, because any cell with substantial expression of actin is positive for SMA. In the breast, myofibroblasts and blood vessels are generally positive for SMA. This becomes problematic in lesions where there are either myofibroblasts or blood vessels in close proximity to the epithelial lesion in question. CD10, the common acute lymphoblastic leukaemia antigen, was originally described as a leukaemia associated antigen expressed in lymphoid precursors and germinal B cells [5,6]. The expression of this marker has been demonstrated in a wide range of non-haemopoietic tissues, including glomerular cells of the kidney, epithelial cells of the prostate gland and small and large intestine, endometrial stromal cells, [7] and MEC of the breast [7-9].

The nuclear stain, p63, a member of the *p53* gene family, shows no cross-reactivity with myofibroblasts or vascular smooth muscle.

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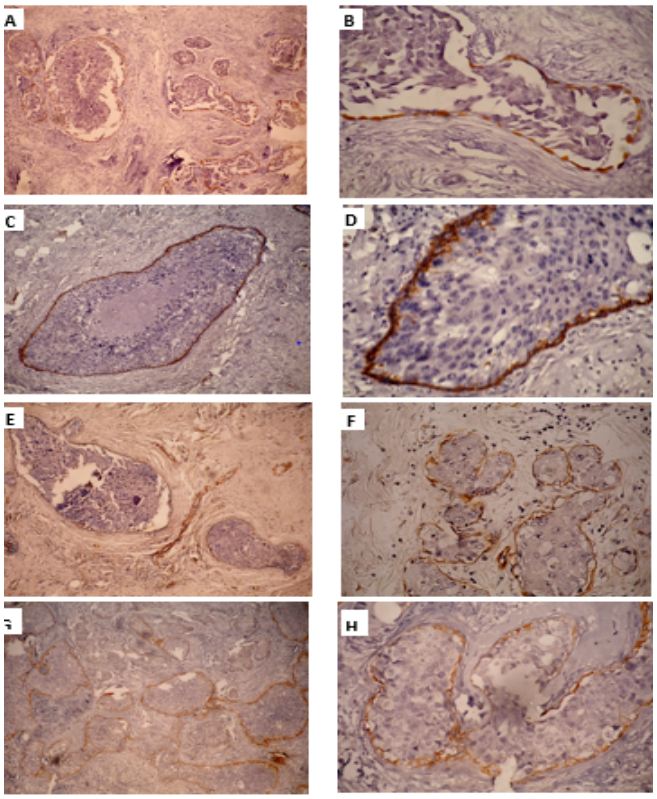

Figure 1: Immunostained sections of breast tumors: (A) nuclear staining of p63 (x100) in myoepithelial cells; (B) p63 (x400); (C) cytoplasmic staining for Smooth muscle actin (x100); (D) Smooth muscle actin (x400); (E) cytoplasmic staining for CD10 (x100); (F) CD10 (x400); (G) cytoplasmic staining for Calponin (x100) (H) Calponin (x400).

It is expressed normally in basal epithelial cells of many organs, including breast, prostate, skin, bladder, and uterine cervix [10]. The myoepithelial cell layer is the sole source of tumor suppressor p63, which is significantly inhibited on proliferation and invasion of associated tumor cells.

Calponin protein is associated with the contractile apparatus in smooth muscle cells. Calponin has been shown to be a useful marker of myoepithelial cells and is considered nearly as sensitive as SMA. But like SMA, staining of myofibroblasts and smooth muscle in blood vessels is seen occasionally causing significant diagnostic difficulties, particularly in the setting of desmoplastic and/or highly vascular stromal responses to both *in situ* and infiltrating malignancy [11].

The purpose of this study was to compare the specificity and sensitivity of four of the most commonly used markers of myoepithelial cells: P63, SMA, CD10 and Calponin.

Material and Methods

Representative paraffin sections from 40 tissue samples of breast masses submitted to the Department of Pathology, Alexandria Faculty of Medicine between 2012 and 2013 were selected for the study. The specimens were either mastectomy or excisional biopsy. The cases were diagnosed as ductal carcinoma *in situ* ± an invasive ductal or lobular carcinoma. The diagnosis of all cases was confirmed by retrieval of pathology reports and review of all hematoxylin and eosin stained sections after taking the approval of the ethics committee in Alexandria Faculty of Medicine.

Immunohistochemical studies

Deparaffinized 4 µm to 5 µm sections of 1 block from each case were rehydrated and subjected to heat-induced epitope retrieval procedures optimized for each antibody. All 4 antibodies, *p63* (dilution 1:1,500; NeoMarkers, Fremont, CA); alpha SMA (dilution 1:100; DAKO); CD10 (dilution 1:1,500; NeoMarkers, Fremont, CA) and calponin (dilution 1:400; DAKO, Carpinteria, CA) were applied to sequential sections from each block. A standard avidin biotin immunoperoxidase technique was used. Sections were stained with 3,3_-diaminobenzidine tetrahydrochlo-ride (DAB), and counterstained with 0.1% Harris Hematoxylin. the nonneoplastic breast tissue that was present in every slide and served as an internal positive control, a separate positive control sample of normal breast tissue was included in each run.

Negative control samples using 10% bovine serum albumin in place of the primary antibody were included in each run as well.

Evaluation of immunohistochemical staining

The MEC layer was identified by the presence of immunoreactivity to P63, SMA, CD10 and calponin in the basal layer of the ductal elements.

First, the reactivity of each antibody was scored semiquantitatively for each of the following elements separately: myoepithelium, myofibroblasts, vascular smooth muscle cells, and tumor (or benign epithelial) cells in each case, and assigned a score of: 0 (negative), 1 (<25% of target cells positive), 2 (26% to 90% of target cells were labeled), or 3 (91% to 100% of target cells positive) [11,12].

Then, the immunohistochemical reaction of the four markers was further evaluated using digital image analysis with a computer-assisted light microscope. The image of each slide was captured using a 40X objective, Images were viewed and recorded using Olympus microscope-equipped with Spot digital camera, using computer program Mat LAB software (image J, the MATHWORKS, inc. USA). The percentage of circumference of the stained acini compared to the overall circumference of the breast acini was calculated to evaluate the sensitivity of the markers in comparison to the originally evaluated sensitivity by subjective methods.

Sensitivity in this context was defined as the ability of the marker to demonstrate myoepithelial cells in deparaffinized, formalin-fixed breast specimens. Specificity is defined as the degree to which the marker distinguishes between myoepithelial cells and other cells likely to be found in the vicinity of the myoepithelial cells, i.e. luminal epithelial cells, vascular smooth muscle cells, and stromal myofibroblasts.

Statistical Methods

Data were analyzed using the Statistical Package for Social Sciences (SPSS ver.20 Chicago, IL, USA). The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, which revealed that the data are not normally distributed, so they were described using median, range. Qualitative data were described using number and percent. Correlation between quantitative variables done using Pearson correlation test. Comparing quantitative variables among the four readings of each case was conducted using Friedman test. Cochrane Q test was used to test binary variables in all statistical tests, level of significance of 0.05 was used, below which the results were considered to be statistically significant.

Results

40 cases of ductal carcinoma *in situ* ± invasive ductal or lobular

carcinoma were analyzed for myoepithelial markers, in all categories of noninvasive breast lesions, antibodies to P63, SMA, CD10 and Calponin were positive on the overwhelming majority of myoepithelial cells Figure 1.

No differences were noted in the immunostaining of low-grade versus high-grade DCIS. In a small minority of cases, however, the myoepithelial cells showed incomplete positivity (score of 2) with the antibodies, resulting in apparent gaps in the myoepithelial cell layer, seen as a "discontinuous" positive signal around the nests of DCIS , defined as spaces equivalent to two or more nuclei between two positive nuclei.

Quantitative analysis indicated that antibodies to calponin yielded a slightly higher sensitivity than those to either P63, CD10 and SMA (65% vs 54%, 19% and 17%, respectively). This difference was statistically significant. Table 1 both semiquantitative assessment and computerized image analysis were statistically correlated.

Specificity Results are summarized in Table 2. Figure 2 Antibodies to p63 showed the highest specificity for myoepithelium of all antibodies

Myoepithelium			
	% positive	Median semiquantitative	Median Computer image analysis
P63	100%	54.5 (17-98)	69.5 (49-96)
CD10	100%	19.5(12-97)	52(27-89)
Calponin	100%	65(15-98)	75(32-93)
SMA	100%	17.5(10-74)	50(33-79)
		P value <0.001*	P value <0.001*

Table 1: Differences between the expression of SMA, CD10, P63 and Calponin in myoepithelial cells (semiquantitative and computerized) and in non-myoepithelial elements in the 40 studied cases of breast tumors.

	Frequencies		p
	-ve	+ve	
P63_non-myoepithelial	29	11	
Calponin_non myoepithelial	10	30	<0.001*
CD10_nonmyoepithelial	21	19	
SMA_nonmyoepithelial	0	40	
*by Cochrane Q test			

Table 2: Difference in expression of the 4 markers in non-myoepithelial layer frequencies.

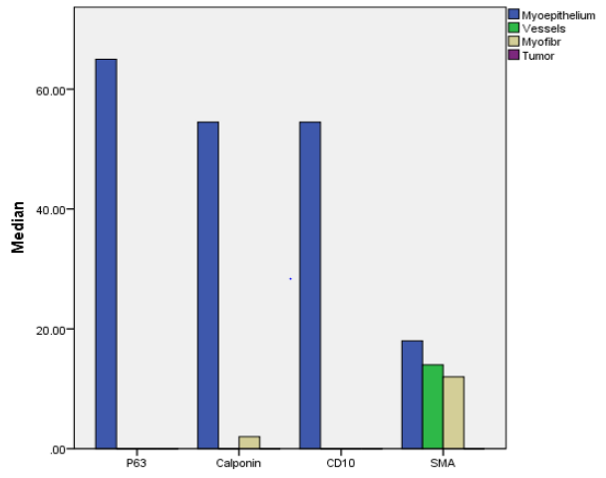

Figure 2: Represent difference in expression of 4 antibodies in myoepithelial and non-myoepithelial cells.

tested, and the least expression in non-myoepithelial layer. In contrast, SMA showed the least specificity and highest non myoepithelial expression especially in stromal myofibroblasts and in vascular smooth muscle cells (Figure 1H).

Discussion

The morphologic distinction between either benign and malignant, or *in situ* and invasive malignant disease of the breast can be problematic, particularly in the setting of core needle biopsies. Although morphology alone can yield accurate diagnosis in the vast majority of breast biopsies and excisions, the presence of considerable inter observer disagreement in the interpretation of difficult lesions based on histology alone was documented [12,13].

Breast ductal and lobular structures are both composed of a double cell layer (an inner, luminal cell and an outer myoepithelial cell), [7] and it has been conclusively demonstrated in a series of investigations over the past 20 years that the presence of an intact peripheral myoepithelial cell layer characterizes all normal and benign breast lesions (e.g. adenosis, papilloma) as well as ductal carcinoma *in situ* (DCIS). Loss of this outer myoepithelial layer is the hallmark of invasive carcinoma, and demonstration of this loss has been documented by immunohistochemical techniques. Researches aim at identifying the ideal marker for myoepithelial cells with absolute sensitivity and specificity [14].

The purpose of this study was to compare the specificity and sensitivity of four of the most commonly used markers of myoepithelial cells: P63, SMA, CD10 and Calponin. Sensitivity in this context was defined as the ability of the marker to demonstrate myoepithelial cells in deparaffinized, formalin-fixed breast specimens. Specificity is defined as the degree to which the marker distinguishes between myoepithelial cells and other cells likely to be found in the vicinity of the myoepithelial cells, i.e., luminal epithelial cells, vascular smooth muscle cells, and stromal myofibroblasts.

In the present study, Calponin and P63 were the most sensitive myoepithelial markers compared to CD10 and SMA with Calponin slightly more sensitive than P63, as assessed by both semiquantitative and computerized image analysis techniques. This finding is in accordance with werling et al. who reported P63 as the most sensitive and specific myoepithelial marker with only a disadvantage of interrupted attaining pattern [11]. Kalof et al. also reported that SMMHC is more sensitive marker for myoepithelial cells than CD10 [15]. In concordance with Cheryl et al. two immunohistochemical myoepithelial markers should be used specially in papillary breast lesions, and they suggested either Calponin with SMMHC or with P63 because they are the most sensitive myoepthelial markers [10]. Moritani et al. [16] reported SMA expression in myoepithelial cells and adjacent stromal spindle cells. Expression of SMA in vessel wall cannot differentiate DCIS surrounded by stromal artificial spaces from vascular involvement and CD10 is better marker for myoepithelial cells with weaker background stromal staining than SMA, also concluded that CD10 was uniformly positive in MEC of normal breast and may serve as a useful marker of breast MEC in difficult breast lesions (for example, sclerosing adenosis versus tubular carcinoma) [16].

And so, SMA should not be used alone as myoepithelial marker due to the decreased specificity. One pitfall is the presence myofibroblasts within desmoplastic stroma adjacent to nests/glands of invasive carcinoma being misinterpreted as myoepithelial cells, resulting in a false-negative diagnosis.

The myoepithelial cell layer is the sole source of tumor suppressor p63, which is significantly inhibited on proliferation and invasion of associated tumor cells. However, Werling et al. [11] noted that it can show at least focal positivity of luminal epithelial cells in a minority of cases.

Calponin offers the highest sensitivity and specificity for myoepithelial cells and should be considered the current "gold standard" for myoepithelial cell identification in breast lesions [11].

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