Applications of Immunohistochemistry in Distinguishing Breast Cancer and Proliferative Lesions

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Abstract: We studied 50 intraductal proliferative lesions and 60 ductal carcinomas in situ, (DCIS), for immunohistochemical expression of ADH-5, cytokeratin (CK) 5/6, CK14, and CK34\textbeta E12. The immunoscore was compared between the two groups. Cytokeratin expression was corroborated by confocal microscopy. ADH-5, CK5/CK6, CK14, and CK34\textbeta E12 showed higher immunoscores in intraductal proliferative lesions than DCIS (P<0.05, respectively). A cutoff immunoscore threshold of 50 appeared discriminatory between proliferative lesions and DCIS, with ADH-5, CK5/CK6, CK14, and CK34\textbeta E12 correctly predicting respectively, The sensitivity of an immunoscore of 50 or less in the diagnosis of DCIS was 98.3%, 91.7%, 85.0%, and 61.7% for ADH-5, CK5/CK6, CK14, and CK34\textbeta E12, respectively, while the specificity was 98.6%, 86.5%, 94.9%, and 84.1%, respectively. ADH-5, CK5/CK6 and CK14 immunohistochemistry can aid in evaluating intraductal proliferative lesions. CK34\textbeta E12 does not appear as useful in identifying DCIS. The combined detection of ADH-5 can be helpful in distinguishing ductal hyperplasia from ductal carcinoma in situ.

Keywords: breast cancer; intraductal proliferative lesions; immunohistochemistry; cytokeratin; marker.

1. Introduction

Intraductal proliferative lesions are a group of cytologically and architecturally diverse proliferations, typically originating from the terminal duct-lobular unit and confined to the mammary duct lobular system\cite{1}. They are associated with an increased risk, albeit of greatly different magnitudes, for the subsequent development of invasive carcinoma\cite{1,2}. Breast cancer is the most frequently diagnosed cancer worldwide\cite{3}. Epithelial proliferations are the most common source of diagnostic difficulty, and they have provided fertile ground for exploration of the potential benefits of immunohistochemistry\cite{4-6}. The use of immunohistochemistry in diagnostic breast pathology are presented in the differentiation of usual ductal hyperplasia from ductal carcinoma in situ\cite{4,7}. Immunohistochemical markers are now commonly used to distinguish benign and in situ proliferations from invasive carcinoma\cite{5,7-10}. The approach takes advantage of the fact that like normal ducts, almost all benign breast lesions and in situ carcinomas have a peripheral layer of myoepithelial cells and basement membrane. High molecular weight cytokeratins have been investigated as potential myoepithelial markers. In particular, cytokeratin 5 has been reported to be a highly specific myoepithelial marker in the differentiation of in situ from invasive carcinomas\cite{9,11}. Its specificity in other contexts, though, is limited by variable staining of luminal epithelial cells and strong positivity in usual ductal hyperplasia. High molecular weight cytokeratins also have a low sensitivity for myoepithelial cells, which hampers their diagnostic utility. Cytokeratins (CKs) are generally thought to be the most fundamental markers of epithelial differentiation because the specific composition of CKs in epithelial cells reflects both cell type and differentiation status. The use of CKs in the distinction of benign epithelial proliferations from DCIS has been previously investigated, with the majority of these studies concluding a utility of CK5/CK6, CK34\textbeta E12, and CK5 and CK14 in distinguishing usual epithelial hyperplasia from atypical ductal hyperplasia (ADH) and DCIS\cite{12}. One key histologic feature that separates benign from DCIS is also the presence of myoepithelial cells, which are preserved in the former and scant or absent in the latter. The preservation of myoepithelial cells in papillomas has been documented using antibodies to muscle actin and CK34\textbeta E12, with CK34\textbeta E12 considered a less specific myoepithelial cell marker, p63, a homologue of p53, is involved in many key developmental events and is expressed in the basal epithelia of multiple organs.
In this study, we detail the expression of four varieties of cytokeratin antibody preparations, ADH-5 (recognizing CKs5, 14, 7, 8 and P63), CK5/CK6, CK14, and CK34βE12 (recognizing CKs 1, 5, 10, and 14) in a series of breast intraductal proliferative lesions, with the aim of determining their role in differentiating the benign proliferative from atypical ductal hyperplasia (ADH) and the malignant in situ carcinoma.

2. Materials and methods

2.1. Patients and Tumors

This study was approved by the Rizhao People's Hospital Ethical Committee. Written informed consent was obtained from all patients before their participation in this study. Histologic material from 110 excision biopsies of breast lesions110 lesions (50 intraductal proliferative lesions and 60 ductal carcinomas in situ, DCIS) diagnosed at Rizhao People's Hospital, between 2003 and 2012, were used for this study. Women with intraductal proliferative lesions were aged 22 to 67 years (mean, 47 years) while those with DCIS ranged from 26 to 88 years (mean, 59 years). Among the intraductal proliferative lesions, there was epithelial hyperplasia of mild to florid degree. Diagnostic review and assignment to intraductal proliferative lesions and DCIS were based on established guidelines of WHO classification. All patients signed informed consents, and this study was approved by the ethics committee of Rizhao People's Hospital.

2.2 Immunohistochemistry

A Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were deparaffinized and rehydrated using standard procedures. ADH-5 Breast Marker Cocktail (Atypical Ductal Hyperplasia) is composed of 5 antibody cocktail in one CK5+CK14+p63+CK7+ CK18 antibodies (Biocare Medical). The conjugated goat anti-mouse polymer horseradish peroxidase(HRP) and the conjugated goat anti-rabbit polymer alkaline phosphatase (AP)secondary antibodies react with both heavy and light chains on mouse and rabbit IgG respectively. This innovative HRP- and AP-polymerization technology provides a significant increase in staining sensitivity when compared to other conventional HRP- or AP-conjugated secondary antibodies. Avidin-biotin blocking procedures are not necessary when using the MACH 2 conjugated secondary antibodies. Avidin-biotin blocking procedures are not necessary when using the MACH 2 conjugated secondary antibodies. It is specially designed for a rapid double stain procedure. An antibody cocktail with a mouse monoclonal and a rabbit The myoepithelium of breast cancer was stained (CK5/14 and/or p63) (DAB) and glandular stained with CK7/18 (Fast Red). This single application can also distinguish hyperplasia of the usual type versus atypical hyperplasia, and distinguish micro invasive and basal phenotypes in most breast cancers. The CK antibodies, dilutions, and antigen retrieval methods are used according to the directions. Immunoreactions of CK5/CK6, CK14, and CK34βE12 were processed using the UltraSensitiveTM S-P Kit (Maixin-Bio, China) according to the manufacturer's instructions, and signals were visualized using the DAB substrate, which stains the target protein yellow. Normal ducts and ductules in the breast tissues served as internal control. For each batch run, a slide from a case specimen was used as a negative control by omitting the primary antibodies[13].

ADH-5 pretreatment Protocol: Heat Retrieval Method: Preheat the retrieval solution to 95°C for 30 minutes in Biocare’s Decloaking Chamber, place, slides into the preheated solution and retrieve under pressure at 95°C for 40 minutes. Alternatively, steam tissue sections for 45-60 minutes or use a wate at 95°C for 40 minutes. Allow solution to cool for 20 minutes then wash in diswater.

Peroxide Block:
Block for 5 minutes at RT.

Protein Block:
Optional: Incubate for 5-10 minutes at RT.

Primary Antibody:
Incubate for 30 minutes at RT.

Double Stain Detection:
Incubate for 30 minutes at RT using Biocare's Multiplex Kit 2.

Chromogen (1):
Incubate for 5 minutes at RT when using Biocare's DAB.

Chromogen (2):
Incubate for 20 minutes at RT with Biocare's Fast Red. Rinse in deionized water.

Counterstain:
(1) Rinse with deionized water.
(2) Incubate for 5 minutes with automated Hematoxylin.
(3) Rinse with TBS Buffer for 1 minute followed by a rinse with deionized water.
2.3 Scoring of Sections

Each section was examined under 4× to 40× objectives of a light microscope after immunostaining. The staining intensity was defined as follows: 0, no staining; 1+, weak; 2+, moderate; 3+, strong. Quantification of positivity (0%-100%) was based on an estimate of the percentage of stained tumor cells in the lesion, with no deliberate distinction made between myoepithelial or luminal epithelial cells that were reactive, as in many instances, it was difficult to unequivocally separate them on light microscopy. A final immunoscore was obtained by multiplying the staining intensity with percentage positivity, thereby giving immunoscores ranging from 0 to 300. The cases were then categorized into four groups showing negative or low (0-50), moderate (51-100), high (101-200), and very high (201-300) scores.

2.4 Statistical Analysis

All statistical analyses were conducted using SPSS (version 17.0). The mean of staining intensity, percentage positivity, and immunoscores were determined. Mean immunoscores for the individual CKs were obtained from the sum of all the immunoscores divided by the number of cases of proliferative lesions and DCIS, respectively. Student’s two-tailed t test was used to examine any differences in immunoreactions between the two sample groups. A P value of 0.05 defined a statistically significant result. Sensitivity, Specificity, Positive and Negative Predictive Values were calculated for each CK, based on the total cohort of 110 cases using the following definitions: sensitivity and specificity referred to the proportion of proliferative lesions and DCIS correctly categorized on immunoscores, respectively. Positive predictive value was the probability that a CK immunoscore of ≤50 could correctly assign a DCIS, whereas the negative predictive value was the likelihood that an immunoscore >50 was able to accurately predict an intraductal proliferative lesion.

3. RESULTS

3.1 Immunoreactivities of ADH-5, CK5/CK6, CK14, and CK34βE12

Immunoreactions for all CK antibodies were localized in the cytoplasm. Normal ducts and ductules present in the tissues served as internal positive controls. The immunoprofiles of ADH-5, CK5/CK6, CK14, and CK34βE12 in intraductal proliferative lesions and DCIS. CK5/CK6 and CK14 exhibited immunostaining mainly in the outer myoepithelial cells. CK7, 18 and CK34βE12, however, showed immunoreactivity in luminal epithelial cells. For CK5/CK6, most proliferative lesions revealed moderate to high immunoscores. p63 was localized to the nucleus, positive staining of myoepithelial cells results in a discontinuous “dotted line” pattern around benign glands and in situ carcinomas. The gaps between positive nuclei are augmented when the myoepithelial layer is attenuated, as is seen in some in situ carcinomas.

Statistical t tests performed on staining intensity, percentage positivity, and immunoscores for ADH-5, CK5/CK6, CK14, and CK34βE12 found all three parameters to be significantly higher in proliferative lesions than DCIS.

In proliferative lesions CK14 stained the largest percentage of cells, with a mean percentage positivity of 83.3% followed by CK34βE12, which had a mean percentage positivity of 50%. CK5/CK6 stained the lowest percentage of tumor cells, having a mean percentage positivity of 47.8%. The percentage positivities of the CK antibodies also varied in DCIS. CK5/CK6 stained an average of 6.0% of tumor cells and CK14 stained 11.8%. CK34βE12 had the highest percentage positivity of stained DCIS tumor cells.

A 2-tailed paired t test was used to compare the percentage positivities of the three CK markers in a pairwise manner. In proliferative lesions, ADH-5, CK14 stained significantly more cells than CK5/CK6 and CK34βE12 (P<0.01). In DCIS, ADH-5, CK5/CK6 stained significantly less cells compared with CK14 and CK34βE12 (P<0.01). The differences in percentage positive staining between CK5/CK6 and CK34βE12 for proliferative lesions, and CK14 and CK34βE12 for DCIS, were not significant (P=0.5, P=0.05, respectively).

3.2 Sensitivity, Specificity, and Positive and Negative Predictive Values

All cases (total of 110 cases) were checked to obtain the sensitivity, specificity, and positive and negative predictive values for ADH-5 and the three CKs investigated in this study, using an immunoscore threshold of 50 to distinguish between proliferative lesions and DCIS. The sensitivity of an immunoscore of 50 or less in the diagnosis of DCIS was 98.3%, 91.7%, 85.0%, and 61.7% for ADH-5, CK5/CK6, CK14, and CK34βE12, respectively, while the specificity was 98.6%, 86.5%, 94.9%, and 84.1%, respectively. ADH-5, CK5/CK6 and CK14 immunohistochemistry can aid in evaluating papillary breast lesions. CK34βE12 does not appear as useful in identifying papillary DCIS.
4. Conclusions

In ADH-5, CK5/CK6 and CK14 immunohistochemistry can aid in evaluating intraductal proliferative lesions. CK34βE12 does not appear as useful in identifying DCIS. The combined detection of ADH-5 (recognizing CKs 5, 14, 7, 8 and P63) can be helpful in distinguishing ductal hyperplasia from ductal carcinoma in situ.

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