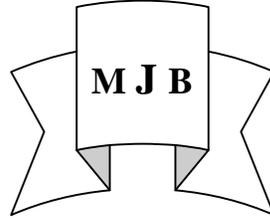# Study of Cytokeratins Markers in Breast Carcinomas

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Abstract

While several prognostic factors have been identified in breast carcinoma, the clinical outcome remains hard to predict for individual patients. Better predictive markers are needed to help guide difficult treatment decisions. In previous studies of breast carcinoma specimens, an association between poor clinical outcome and the expression of cytokeratin markers 17 and/or cytokeratin 5 mRNAs was noted. Here we describe the results of immunohistochemistry studies using monoclonal antibodies against these markers to analyze 55 paraffin-embedded breast tumors. We found that expression of cytokeratin markers 17 and/or cytokeratin 5/6 in tumor cells was associated with a poor clinical outcome. Moreover, multivariate analysis showed that in node-negative breast carcinoma, expression of these cytokeratins markers were a prognostic factors independent of tumor size and tumor grade.

الخلاصة

بالرغم من وجود عدة عوامل تكهنية مستقبلية لسرطنة الثدي لكن نتائج مستقبل المرض سريريا بقي صعب التكهن في حالات المرض الفردية مما استدعت الظرورة استعمال معلمات ورمية جديدة للمساعدة في توجيه القرارات العلاجية الصعبة. في دراسات سابقة لسرطنة الثدي، وجدت علاقة بين النتائج السريرية الفقيرة ووجود معلمات الكيراتين الورمية. في البحث الحالي درست معلمات السايتوكيراتين الورمية باستخدام طريقة الامينوبيركسديز المناعية في 55 حالة لسرطنة الثدي. اظهرت النتائج وجودالمعلمات الورمية لسايتوكيراتين (17، 5\6) في خلايا سرطنة الثدي وان لها علاقة بسوء المستقبل السريري لهذه الحالات، اضافتا على ذلك فا ن التحليل الاحصائي المتعدد الاوجه للنتائج بين ان في حالات السرطنة غير المصاحب للانتشار في الغدد اللمفاوية كانت لمعلمات السايتوكيراتين الورمية عوامل تكهنية مستقبلية مستقلة وغير مرتبطة بحجم الورم او درجته الخبيثة.

Introduction

A number of parameters are used to predict the clinical outcome of breast carcinoma, and to guide treatment decisions accordingly. The most important prognostic factors in current use are clinical features such as lymph node (LN) status, tumor size, and tumor grade. The expression level and staining patterns of several proteins are also useful in predicting which tumors will respond to specific therapies; tamoxifen is used to treat only estrogen receptor-positive tumors and herceptin to treat Her2/neu over expressing tumors. Although these histological prognosticators are undeniably useful, the clinical course of any individual patient with breast carcinoma remains

difficult to predict. There is little doubt that there is still considerable molecular heterogeneity within the existing tumor categories. Many studies have continued to identify and explore molecular markers that might help to better stratify patients. In multivariate analysis, however, many of these factors co-vary and are therefore not independently informative.[1, 2]

With the development of DNA microarray technologies for large-scale analysis of gene expression patterns, a systematic genome-wide search for molecular markers in breast carcinoma has become possible.[3-5] recently genomic expression patterns in breast carcinoma specimens using DNA microarrays were analyze[6] .Five groups of patients that could be distinguished on the basis of their global gene expression patterns were identified. Two traditional markers, Her2/neu and the estrogen receptor, and a group of cytokeratin genes, were notable for their differential expression among the breast cancer subgroups. Analysis of the survival data in the study showed that two subgroups had a significantly poorer prognosis; one was characterized by elevated expression of Her2/neu, the other was characterized by high levels of expression of genes characteristic of the basal epithelial cells of the normal mammary gland, including the genes that encode cytokeratins markers 17 and 5., we used the recently developed technique of tissue immunohistochemistry (TMA) in a retrospective evaluation of breast tumor samples to explore its clinical significance [7,8]. This approach allowed us to evaluate protein expression in many tumors at once, with a single staining reaction on a single glass slide. Using commercially available antibodies

against cytokeratin markers 17 and 5/6, we carried out an immunohistochemical assay for these markers on sample from 55 breast carcinomas.

Materials and Methods

A total of 55 different paraffin-embedded breast carcinoma samples were studied .The histological parameters for all cases were examined and the histological type and grade was determined for each case according to Elston and Ellis.[9] Follow-up was obtained for 55 cases and ranged from 6 to 120 months. After surgery, 31 patients received additional systemic therapy .Tissue immunohistochemistry (TMA) were constructed by obtaining 0.6-mm diameter tissue cores from each tumor and placing these cores in a new paraffin block in rows and columns.[7,8,10,11] Each of the 55 cases was sampled twice, once from the center of the tumor, and once from the periphery of the mass. A more detailed description of tumor tissue immunohistochemical assay has been published previously.[7,8]

Immunohistochemical Scoring

Double-staining of normal breast epithelium and tumors in conventional paraffin sections was performed by first staining luminal cells with CAM5.2 using alkaline phosphatase/fast blue staining and subsequent staining of basal cells with CK17 using horseradish peroxidase/DAB staining. Sections of tissue were stained with monoclonal antibodies specific for cytokeratin markers 17 (DAKO, dilution 1:10) and cytokeratin 5/6 (dilution 1:10) after antigen retrieval by microwave treatment in citrate buffer.[12] Staining results were scored as follows: 1, invasive tumor cells present in tissue core and no staining seen; 2, invasive tumor cells present and weak staining; 3, invasive

tumor cells present with strong staining. Only those cores showing invasive carcinoma were included in the outcome analysis. Cases that either had no tissue present on the array sections or cases, in which the material sampled consisted only of fat, fibrosis, normal mammary glands, or in situ carcinoma, were omitted from further analysis. Immunohistochemical staining for cytokeratins markers often produced only focal staining of tumor cells. A breast tumor sample was scored as staining positive for the keratins if infiltrating carcinoma in one or more of the cores from that sample reacted with either of the antibodies. To aid in recognizing infiltrating carcinoma in the core samples, sections were also stained with a cytokeratin mix reacting with cytokeratins 8 and 18 (dilution 1:20) after antigen unmasking by trypsin digestion to highlight invasive carcinoma cells. Sections were also stained with antibodies against estrogen receptor (DAKO), and Her2/neu (DAKO). Scoring for Her2/neu was performed following approved manufacturer's instructions. Nuclear staining was scored for estrogen receptor (ER) staining, with tumors with less than 5% nuclear staining scored as negative, those with 5 to 20% staining as weak, and those with more than 20% staining scored as strongly positive.

Rabbit Antiserum

 A rabbit polyclonal antiserum was raised by injecting three peptides derived from cytokeratin markers 17 protein sequence. The peptides were conjugated to KL hemocyanin (KLH). The peptide-KLH conjugate was injected into two out-bred rabbits. The serum was harvested after the rabbit's demonstrated significant anti-peptide titer. Affinity-purified antiserum was obtained by binding the antiserum to an affinity column conjugated with the three peptides; the bound antibodies were eluted with a pH gradient.

Statistical Analysis

 Univariate (Kaplan-Meier) analysis of patient survival for subgroups defined on the basis of cytokeratin expression was performed. Subsequent multivariate analyses were performed using Cox's proportional hazards model for survival data.[13]

Results

 Basal Keratin Staining in Normal Breast and Breast Carcinoma

In normal breast, CK17 and CK5/6 stain the basal layer of breast ductal epithelium while keratins 8 and 18 stain luminal cells (Figure 1A). An examination of whole paraffin sections of breast carcinoma showed that cytokeratin markers 17 and 5/6 expression in paraffin-embedded tissue, when present, was focal (Figure 1B) with often less than 10% of tumor cells reacting. To further investigate the focal reactivity of the monoclonal antibodies against the basal-type cytokeratins, and as an attempt to improve the reliability of this test, we raised a rabbit antiserum against CK17. This antiserum was tested with breast samples. The new antiserum and the monoclonal antibody against CK17 showed very similar reactivity with epithelial cells in the breast sections. Both reagents stained the same fraction of tumor cells suggesting that neither is a significantly better reagent. These results suggest that the focal reactivity seen with monoclonal anti-CK17 was not due to weak reactivity of the monoclonal antibody but to the expression of this basal keratin in only a subset of tumor cells at a level detectable by immunohistochemistry. After

combining results from the peripheral and central area, 7 and 6 tumors scored positive (either weak or strongly) for CK17 and CK5/6, respectively. By combining the results from the stains for CK17 and CK5/6, 10 cases of the 55 tumors examined reacted with either CK17 and/or CK5/6. Of these 10 cases, 5 stained for both antibodies, while the remainder reacted with either of the two antibodies. Follow-up data were available for 50 cases on which CK staining data were obtained. The follow-up period ranged from 6 to 120 months with a mean of 60 months .Kaplan-Meier survival analysis for all patients with follow-up showed that the absence of detectable cytokeratin markers 17 or cytokeratin 5 was associated with a significantly better prognosis than the presence of either of these cytokeratins (Figure 2A, P= 0.012). The lymph node status was known in 47 patients. In the group of 30 patients with known lymph node metastases, the expression of CK17 and CK5/6 had no predictive value. In contrast, in the group of 24 patients without lymph node metastases at presentation, CK17 and/or CK5/6 expression was associated with significantly shorter survival (Figure 2B, $P = 0.006$). The percentage of basal keratin-positive tumors was similar in patients with and without lymph node metastases. Multivariate analysis on all patients taken together showed that the prognostic association of basal cytokeratin expression with poor outcome was not independent from tumor size, LN status, and histological grade. When only patients who presented without lymph node metastases were considered, however, the expression of basal cytokeratins was not only a significant prognostic factor, but its prognostic significance was

independent of tumor size, tumor grade, Her2/neu status, ER status.

Her2/neu and Estrogen Receptor Staining on Breast Carcinoma immunohistochemistry

Sections of the tissue immunohistochemistry made with peripheral cores were stained for estrogen receptor and Her2/neu. As expected, expression of estrogen receptors was associated with a better clinical outcome. This finding was independent of tumor grade, LN status, and size. In contrast, Her2/neu expression was associated with a poor prognosis. These results are compatible with previous reports and are similar to those of two prior studies[7, 8] conducting using the same breast tumor tissue immunohistochemistry used here.

We also stained sections with antibodies specific for GATA-binding protein 3, a protein that has previously been observed to be co-expressed with the estrogen receptor.[14] Expression of GATA-3 was associated with a good clinical outcome, and highly correlated ($\chi^2 = 720.3$) with estrogen receptor expression. Our immunohistochemical staining results for estrogen receptor and Her2/neu thus confirm results of prior studies, and provide further evidence of the utility and reliability of tissue immunohistochemical -based studies. A comparison was made between the basal cytokeratin staining and Her2/neu staining on tumor tissues for which both cytokeratin and Her2/neu staining data were available. No statistically significant correlation was found between basal cytokeratin staining and Her2/neu expression, a finding further confirmed by our multivariate analysis .

Discussion

The sequencing of the human genome and the development of massively parallel technologies for analyzing gene expression have opened a new era of molecular diagnostic medicine. DNA microarray analysis now allows the rapid determination of mRNA levels for many thousands of genes in tumor samples while tissue microarrays can be used to analyze large numbers of tumors by immunohistochemical or other staining methods. Perou et al examined the molecular profiles of breast carcinomas from 42 patients using DNA microarrays representing more than 8000 genes[15] .That study identified a subset of carcinomas that was distinguished from the other tumors by their relatively high level of expression of a specific set of genes characteristic of the basal epithelial cells of normal mammary ducts. Basal keratin expression distinguished this set of tumors from the majority of the tumor samples, which expressed keratin types typically expressed in normal luminal breast epithelial cells. A subsequent study on a larger group of 78 breast tumors showed that the carcinomas in which these "basal" keratins were expressed had a significantly poorer prognosis.[1]

Using tissue immunohistochemistry with 55 breast carcinoma cases, we show here that the presence of basal epithelial cytokeratins markers in breast carcinoma cells is associated with a poor prognosis. Because of the relatively small size of the tissue samples available for analysis in tissue, the interpretation of stains that are only focally or heterogeneously reactive can be ambiguous. This was a special concern in our study, because the expression of basal keratins, as detected by immunohistochemical staining, is often very localized, with only scattered tumor cells in a cross-section of the tumor mass showing detectable reactivity. To minimize this ambiguity, we combined results from samples taken from two different areas (central and peripheral) of each tumor, and stained samples from each site with two different antibodies (anti-CK17 and anti-CK5/6). Sixteen percent of the tumors we examined expressed detectable CK17 and/or CK5/6. This frequency was similar to that found in an independent patient population, in which high levels of mRNA for cytokeratins 5 and 17 were detected in 18% of the breast carcinomas studied. In both studies the expression of these markers was associated with poor clinical outcome[.6,15]

Because of the large number of patient samples that we were able to analyze using tissue immunohistochemistry, we were able to test separately the prognostic significance of these markers in patients with lymph node metastases and those without evident metastases. In patients with metastatic disease to the lymph nodes, the expression of the basal cytokeratins markers was not associated with a significant difference in clinical outcome. However, in patients without detectable lymph node metastases, expression of "basal" cytokeratins markers was associated with a poor prognosis independent of tumor size, tumor grade, or immunostain reactivity for ER, or Her2/neu,. These findings support the idea that anti-cytokeratin antibodies may identify a distinct form of breast cancer, derived from basal cells rather than the luminal cells from which the majority of mammary cancers appear to arise. Further studies are needed to define the cellular origin of each of these groups of tumors.

Two previous immunohistochemistry studies have suggested a correlation

between basal cell type markers and poor prognosis. Dairkee and colleagues[16] reported four cases of breast carcinoma with expression of the marker 312C8-1 for myoepithelial cells in the tumor cells and noted a poor clinical outcome. In a study of 51 patients with clinical follow-up, Malzahn et al [17] found a statistically significant association of basal/myoepithelial cell keratin expression with poor prognosis. In contrast to our findings, this association was found to be statistically significant in LN-positive patients but not in LN-negative patients.

The interpretation of immunohis-tochemical staining results for the basal keratins is complicated by the focal and often weak reactivity of monoclonal antibodies against these proteins, limiting their use in clinical settings. We have therefore begun searching for better immunohistochemical markers for this group of breast cancers. We considered the possibility that alternative antibodies against these cytokeratins might provide better performance. Analysis of breast carcinoma samples showed that the number of staining cells, the focal staining pattern, and the intensity of staining were similar for a new polyclonal antiserum against CK17 and the commercial monoclonal antibodies. This result suggests that the basal keratins are indeed only focally expressed at a level detectable by immunohistochemistry and that the low numbers of cells stained with antibodies are not due to a weak reactivity of the monoclonal antibodies with the protein. Several studies have now reported that breast cancers expressing basal cytokeratins are not uncommon (>10%), and that they are associated with a poor prognosis[1]. Patients with metastatic breast carcinoma to the axillary lymph nodes are at high risk for recurrence and most receive adjuvant therapy. The situation for "node-negative" patients is less clear; depending on the size and grade of the tumor, the reported recurrence rate varies between five and thirty percent. In patients who present without detectable lymph-node metastases, the clinical decision to give or withhold systemic therapy is therefore a difficult one and hence it is for this group of patients that the need for new prognostic markers is the most acute. The relative size of this group of patients is expected to increase, due to continuing advances in screening and diagnostic techniques that allow detection of smaller breast tumors. Most of these smaller tumors have not metastasized to the "sentinel" lymph node. This group of patients therefore faces a difficult choice among a variety of treatment options, including lumpectomy, mastectomy, chemotherapy, radiation therapy, and hormonal therapy.

Conclusion

Expression of cytokeratins markers appears to define a group of breast tumors with a relatively high mortality rate: clearly a significant consideration in the treatment decisions for node-negative breast carcinoma patients. Whether more aggressive treatment procedures will improve the outcome for these patients, and which of the available options provide the greatest benefit, is an important question for future studies. The challenge in the future will be to develop therapies directed specifically at this molecularly and clinically distinct form of breast cancer.


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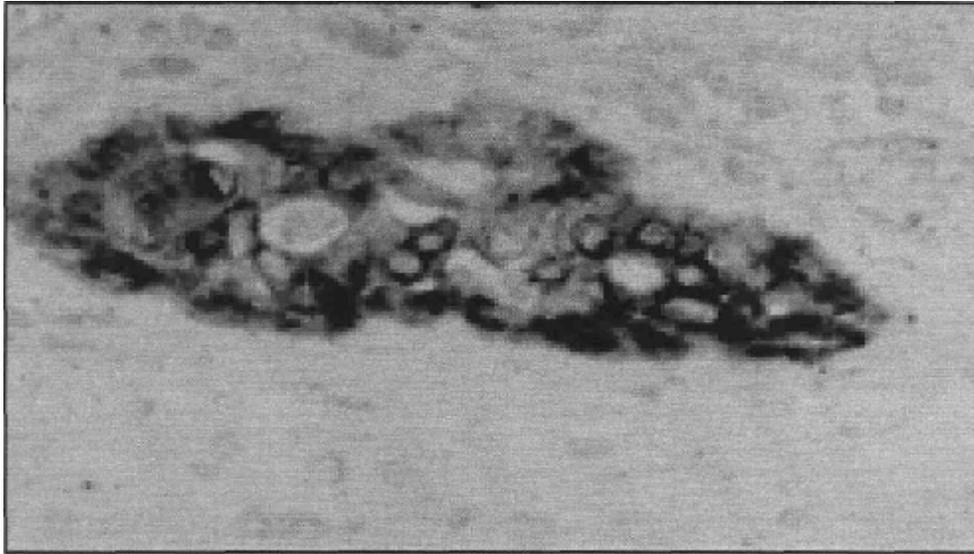
Figure 1. A: Normal mammary gland simultaneously stained with CAMS.2 monoclonal antibody [specific for keratins 8 and 18] (blue), and monoclonal anti-cytokeratin 17 (brown). Note that the CAMS.2 antibody specifically stains the lumenal epithelial cells, while the anti-cytokeratin 17 antibody specifically stains the basal epithelial cells of the normal mammary duct.
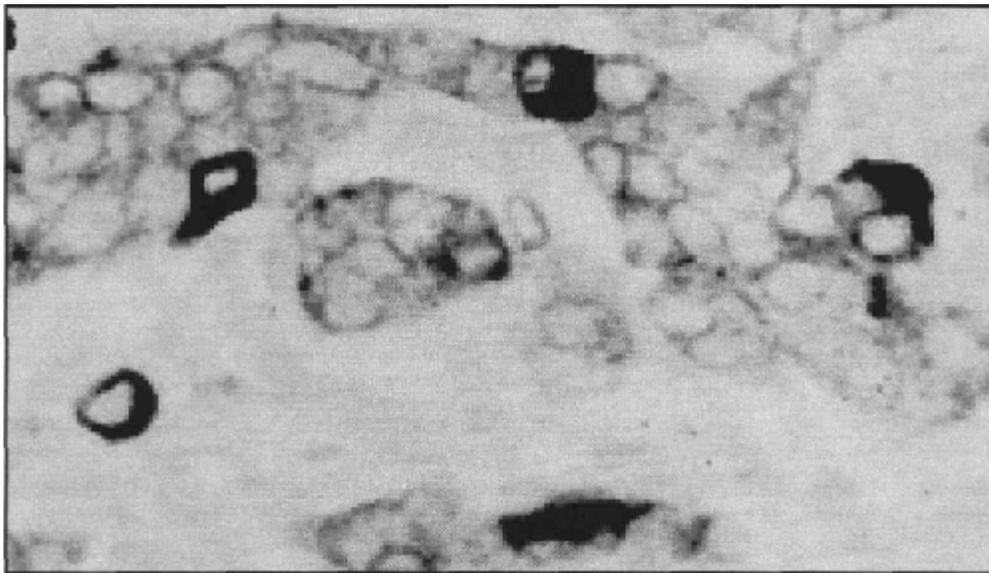
Figure 1. B: Whole paraffin section of breast carcinoma stained with CAM5.2 monoclonal antibody (blue], and monoclonal anti-cytokeratin 17 (brown). Note the focal staining pattern for cytokeratin 17.
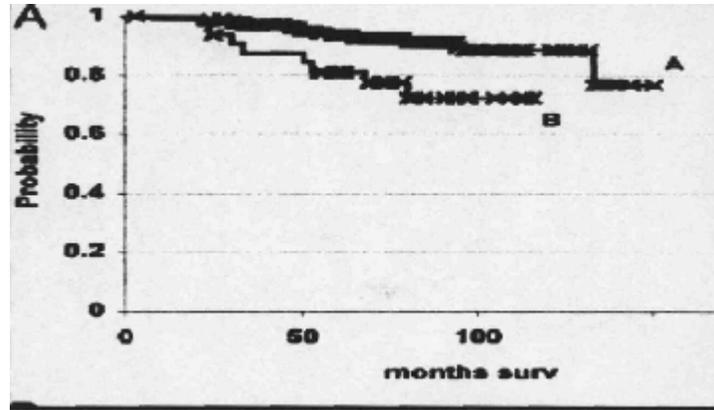
Figure 2. A: Kaplan-Meier survival curve showing poor outcome in cytokeratin 17 and/or 5/6-positive tumors (P = 0.012). Clinical follow-up was available for 55 patients mean, 60 months. **A:** No expression of CK17 or cytokeratin 5/6 in tumor cells. **B:** Expression of CK17 and/or cytokeratin 5/6 in tumor cells.
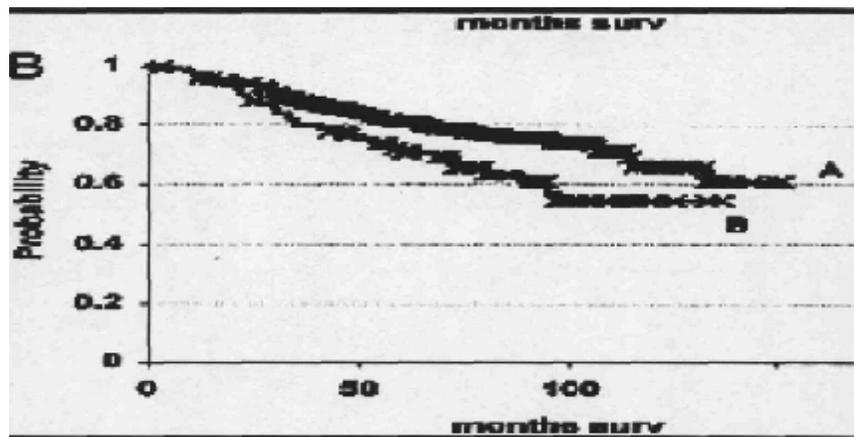
Figure 2. B: The effect of cytokeratin 17 and/or cytokeratin 5/6 expression in 24 patients with negative lymph nodes (P = 0.006). The lymph node status was known in 47 patients. Patients expressing basal keratin (B) in this group have worse outcome than in patients without expression of these markers (A). Multivariate analysis showed that this effect was independent of tumor size, tumor grade, or Her2neu, or ER expression.