Down-Regulated Expression of Cytokeratin 18 Promotes Progression of Human Breast Cancer

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ABSTRACT

Purpose: **Cytokeratins (CKs) have been recognized for >20 years as structural marker proteins specific for epithelial cells. Recent expression profiling analyses indicate, however, that CK down-regulation may occur in breast cancer.**

Experimental Design: **Here we evaluated the expression pattern of CK18 by immunohistochemical analysis of primary breast carcinomas ($n = 1458$) spotted on a high-density tissue microarray. The findings were correlated to histopathological risk factors and clinical outcome.**

Results: **Down-regulation of CK18 (as compared to normal breast tissue) was observed in 25.4% of the tumors with a lower rate in lobular carcinomas (17.0%) than in ductal carcinomas (25.4%) or other histological entities (32.5%). CK down-regulation was significantly correlated to advanced tumor stage and high grade but not to axillary lymph node status. Kaplan-Meier survival analysis revealed CK18 as a prognostic indicator of overall survival ($P = 0.015$) and cancer-specific survival ($P = 0.005$).**

Conclusions: **Down-regulation of the luminal CK18 is not rare and a clinically relevant event in breast cancer. This finding has important implications for the use of CK18 as epithelial tumor marker. The correlations with clinical follow-up suggest that CK18 might suppress tumor progression.**

INTRODUCTION

The majority of cancers in industrialized countries are solid tumors derived from epithelial tissues, such as carcinomas of the breast, lung, gastrointestinal tract, and prostate. Epithelial cell and tissue architecture is largely determined by cytoskeletal filaments, which are part of an intracellular protein network comprising the cytoskeleton itself, junctional complexes at the cell border, and numerous associated proteins. The cytoskeleton of epithelia is predominantly formed by cytokeratins (CK), which are grouped into a type I (acidic, *CK9* through *CK20*) and a type II (neutral-basic, *CK1-CK8*) gene family (1). CKs provide mechanical stability to tissues, as implicated by a range of pathological phenotypes seen in patients bearing mutations in epidermal keratins (2). All of the CKs share the same domain structure and form obligate heteropolymers from any combination of type I and II molecules to built intermediate filaments (3). In various epithelia, they form specific expression pairs of at least one protein member of each type.

Therefore, CKs have been recognized for >20 years as epithelial markers in diagnostic histopathology (4–6). In normal mammary epithelium, luminal cells usually express CK8, 18, and 19, which are typical for simple epithelia (4). Most malignant breast tumors are adenocarcinomas derived from simple breast epithelium, and monoclonal antibodies directed against CK18 have, therefore, been used to identify primary and metastatic breast cancer cells in numerous investigations (4, 7). However, various regulatory changes in CK expression at the transcriptional and post-transcriptional level have been described in experimental studies on epithelial tumor cells, challenging the view that CKs are merely marker proteins (8–10). Additional evidence for a more widespread role of CKs came from mouse gene knockout studies. The double deletion of CK18 and 19 has been shown to result in the complete lack of a functional CK skeleton, which caused embryonic lethality (11).

The question is now whether CK18 plays an important role for tumor progression in cancer patients. Previous expression profiling studies indicated that expression of CK18 is down-regulated in metastatic breast cancers (12, 13). Moreover, we recently used bone marrow as an indicator organ for micrometastatic tumor cells in breast cancer patients and proposed that the onset of primary hematogenous metastasis might be facilitated by down-regulation of luminal cytokeratins including CK18 (14).

On the basis of the in-depth analysis of high-density tissue microarrays (TMAs), we here demonstrate that down-regulation of CK18 expression is not uncommon in primary breast carcinomas and that this phenotype predicts poor clinical outcome. The present findings challenge the value of CK18 as a reliable epithelial marker (15–17) and propose a new role for this protein as a putative suppressor of tumor progression in breast cancer.

MATERIALS AND METHODS

Breast Cancer TMA. For the construction and composition of the breast cancer TMA, H&E-stained sections were made from each paraffin-embedded tissue to allow the identification of representative tumor regions. Tissue cylinders with a diameter of 0.6 mm were then punched from selected areas of

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Table 1 CK18[a] expression of primary breast tumors in relation to histopathological factors

Characteristics of primary carcinomas[b]	CK 18 expression: Normal expression (n = 1087)	CK 18 expression: Partial and complete loss of CK18 expression (n = 371)	P
Histological type			
Ductal carcinoma (n = 1042)	777 (74.6%)	265 (25.4%)	0.002
Lobular carcinoma (n = 188)	156 (83%)	32 (17.0%)	
Other (n = 228)[c]	154 (67.5%)	74 (32.5%)	
Tumor size			
pT1 (n = 497)	394 (79.3%)	103 (20.7%)	0.008
pT2 (n = 676)	483 (71.4%)	193 (28.6%)	
pT3 (n = 94)	64 (68.1)	30 (31.9%)	
pT4 (n = 185)	142 (76.8%)	43 (23.2%)	
Grading			
G1 (n = 330)	284 (86.1%)	46 (13.9%)	0.0001
G2 (n = 566)	452 (79.9%)	114 (20.1%)	
G3 (n = 474)	286 (60.3%)	188 (39.7%)	
Lymph node status			
pN0 (n = 585)	429 (73.3%)	156 (26.7%)	0.640
pN1/2 (n = 620)	462 (74.5%)	158 (25.5%)	
Mitotic index[d]			
1 (n = 693)	580 (83.7%)	113 (16.3%)	0.0001
2 (n = 256)	192 (75%)	64 (25%)	
3 (n = 417)	248 (59.5%)	169 (40.5%)	
Age			
≤ 50 (n = 250)	185 (74%)	65 (26%)	0.855
> 50 (n = 937)	688 (73.4%)	249 (26.6%)	

[a] CK, cytokeratin.

[b] Data from tumor size, lymph node status, grading, and age were not available in 6, 253, 88, and 271 cases, respectively.

[c] This group can be subdivided into medullary carcinoma (n = 50), mucinous carcinoma (n = 46), cribriform carcinoma (n = 37), tubular carcinoma (n = 34), papillary carcinoma (n = 18), apocrine carcinoma (n = 11), clear cell carcinoma (n = 9), atypic medullary carcinoma (n = 6), metaplastic carcinoma (n = 4), neuroendocrine carcinoma (n = 2), and adenoid cystic carcinoma (n = 1). Ten breast carcinoma samples were not histologically specified.

[d] M1 = <10 mitoses per high power field (HPF), M2 = 10–20 mitoses per HPF, and M3 ≧ 20 mitoses per HPF.

each "donor" block using a homemade semiautomated precision instrument and brought into a "recipient" paraffin block containing premade holes (18). The expression of CK18 on primary breast carcinomas was evaluated on the basis of 1458 TMA sections where an interpretable staining could be obtained for CK18. Causes of uninterpretable results included lack of tumor cells or tissue (empty spots), presence of necrotic/damaged tissues in the samples, and unsuccessful staining.

The series included 1042 ductal carcinomas, 188 lobular carcinomas, 228 other breast cancer entities (Table 1), and an additional set of 276 non-neoplastic or precancerous tissues as controls. The tumors were graded as 1, 2, and 3 according to Elston and Ellis (19). The median follow-up period was 51 month (range, 1–150 months) for all of the patients. Written informed consent was obtained from all of the patients in this study.

Immunohistochemical Analysis. Formalin-fixed, paraffin-embedded tumor arrays were deparaffinized and subsequently subjected to a 20-min microwave pretreatment in citrate buffer (10 mM; pH 6.0) Immunostaining was performed on an automated staining machine (DAKO Diagnostika GmbH, Hamburg, Germany) with the mouse antihuman CK18 antibody clone DC10 (DAKO Diagnostika GmbH, concentration 1:100) and the DAKO ChemMate Detection kit. Application of the primary antibody was followed by incubation with biotinylated goat antimouse immunoglobulins (10 min), streptavidin conjugated to horseradish peroxidase (10 min), 3,3′-diaminobenzidine tetrahydrochlorid/H_2O_2 as chromogen, and hematoxylin counterstaining.

The number of CK18-positive cells in each tumor sample of the TMA was estimated by one experienced pathologist (G. S.) in a consecutive analysis of all samples on 1 day to ensure maximal internal consistency. The staining results were grouped into normal CK expression (*i.e.*, ≥90% stained cells), reduced CK expression (*i.e.*, <90% stained cells), and complete loss of CK expression.

The staining intensity was assessed semiquantitatively by using a score from 1 to 3. The strongest staining intensity seen in tumor cells on a particular sample was indicated as 3. We have not included this information in our calculation, because the interpretation of the staining intensities is a more subjective value than the percentage of stained cells. However, the addition of the staining intensity results would not change our findings.

Statistical Analysis. The χ^2 test was used to evaluate the relationship between the CK18 expression and other known risk factors. We constructed Kaplan-Meier life-table curves for cause-related and overall survival. The log-rank test was used to compare significance of differences between the curves. A P-value < 0.05 was considered to indicate a statistically significant difference. All of the tests were two-tailed. The joint effects with already recognized prognostically relevant variables were examined via Cox proportional hazard analysis (threshold for statistical significance was $P = 0.05$). For statistical analyses, we used SPSS software for PC (version 11 for Windows).

RESULTS

Pattern of CK18 Expression in Human Breast Cancer. To analyze the overall incidence of CK18 expression in human mammary carcinomas, an immunohistochemical study was performed, using a breast cancer TMA containing 1458 interpretable tumor samples plus various control tissue sections (18). A representative staining of a breast cancer TMA is shown in Fig. 1.

CK18 was found to be consistently expressed (≥90% stained cells) in normal mammary glands, which served as an internal positive control on the TMA. In contrast, expression of this CK was variable in breast cancer tissues. The staining results were divided into three categories based on the expression on normal breast ductal cells: (*a*) normal CK18 expression, *i.e.*, ≥90% stained tumor cells; (*b*) partial loss of CK18 expression, *i.e.*, <90% stained tumor cells; and (*c*) complete loss of CK18 expression. Normal expression of CK18 (*i.e.*, ≥90% cancer cells were stained) was found in 1087 (74.6%) tumors, and complete loss of CK18 expression on all of the cancer cells

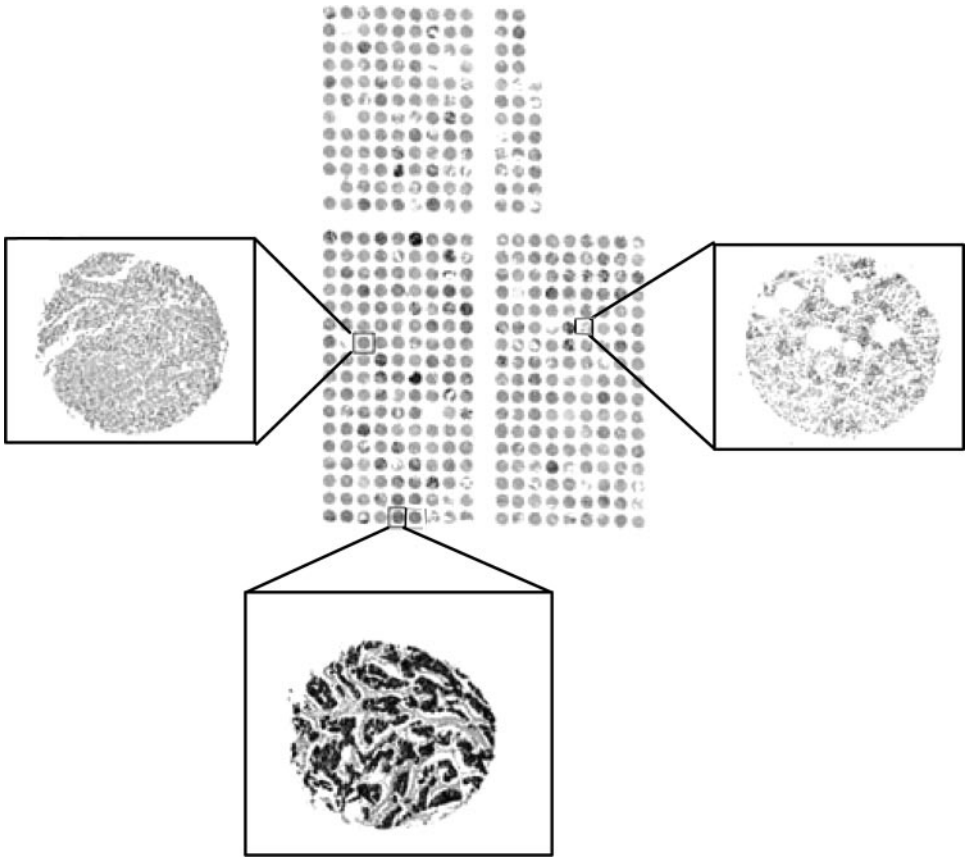

Fig. 1 Representative high-density tissue array stained with a monoclonal antibody against cytokeratin (CK). Overview of a tissue microarray (TMA) and three enlarged tumor spots with normal CK expression (100% stained tumor cells), reduced CK expression (*i.e.*, <90% stained cells), and complete loss of CK expression.

was observed in 170 (11.7%) tumors. In the remaining TMA spots (13.7%), variable fractions of breast tumor cells were stained with the anti-CK18 antibody, indicating a partial loss of CK18 expression.

As shown in Table 1, marked differences between breast cancer entities were observed. The tumor samples with partial and complete loss of CK18 expression (25.4%) were grouped together in comparison to the tumor samples with normal cytokeratin expression. Loss of CK18 expression was significantly less frequent (Table 1) in lobular carcinomas (17.0%) than in ductal carcinomas (25.4%) or other types of carcinomas (32.5%). Loss of CK 18 staining was significantly correlated with high tumor grade, high mitotic index, and with increased tumor size (pT-stage; Table 1). The finding that the incidence of low CK18 expression in pT4 tumors was lower than in pT2 tumors may be partly due to sample size bias. But it must be noticed that pT4 tumors correspond to tumors of any size that are characterized by skin infiltration. Therefore, pT4 tumors cannot be compared directly with pT1–3 tumors. No correlation was found between loss of expression of CK18 analyzed and lymph node status (Table 1). Patients with lymph node metastasis (stage pN1/2) showed a similar pattern of the CK18 expression as patients without lymph node metastases (stage pN0).

Prognostic Significance of CK18 Expression. The patients ($n = 1458$) were followed over an extended period of >10 years, and comparison of survival distributions by Kaplan-Meier analysis using the log-rank test were performed. The analysis showed that partial and complete loss of CK18 staining was associated significantly with a reduced cancer-specific ($P = 0.005$; Fig. 2) and overall survival ($P = 0.015$; data not shown).

To investigate whether CK18 expression represents an independent prognostic factor for breast cancer, a multivariate analysis including the histopathological parameters tumor stage, lymph node status, and grade of differentiation was performed. The data showed that CK18 expression was not an independent prognostic parameter with regard to cancer-related survival ($P = 0.101$; data not shown), but was closely linked to prognostic value of grade.

DISCUSSION

On the basis of the analysis of a high-density TMA, we demonstrated that expression of CK18 is variable in primary breast carcinomas. Partial or complete loss of CK18 expression was observed in ~25% of the tumors depending on their histological type. Thus, the use of the individual luminal CK18 as a diagnostic marker for breast cancer cells might lead to false-negative findings due to the observed down-regulation of this protein. This conclusion is additionally supported by reports demonstrating that a subset of micrometastatic tumor cells present in the bone marrow, as the major site of metastatic relapse in breast cancer, lack an expression of CK18 (20). Taken together, these findings argue in favor of using a mixture of several nonspecific antibodies or a broad spectrum anti-CK

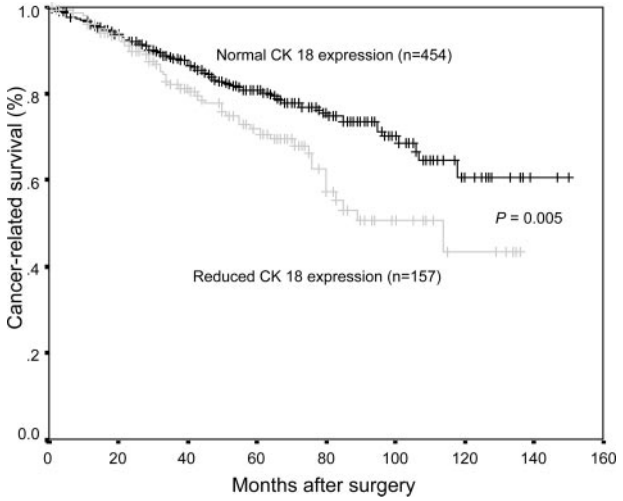

Fig. 2 Kaplan-Meier estimates of cancer-related survival of breast cancer patients, according to the level of cytokeratin 18 expression in the primary tumor. The tumors of the patients are grouped according to 100% stained tumor cells (*normal expression*) and <100% stained tumor cells (*reduced expression*).

antibody directed against a common epitope present on several different CKs for diagnostic purposes, in particular for the detection of occult metastatic cells (20–22).

It is known that particular breast cancer samples possess high intratumoral heterogeneity. The limited ability to detect heterogeneous expression is an inherent weakness of the TMA method. However, previous studies using TMAs of breast cancer tissues have demonstrated that TMAs are highly suited to detect prognostic molecular features (23–25). In one study, TMAs yielded better information on the prognostic impact of p53 staining than large sections analyzed by the same investigator (26). Overall, these data strongly suggest that the high standardization of the staining (all of the samples stained under identical conditions), the objectivity of the analysis (limited to a small piece of tissue), and the high number of tumors more than compensates for the perceived disadvantage of analyzing small tissues only.

Comparison of the CK18 expression pattern with different clinicopathological risk factors revealed highly significant correlations to the size (pT1–3), differentiation grade, and mitotic index of the primary tumor. These parameters are (among other factors) a function of the proliferation rate of the primary tumor, suggesting a possible relationship between down-regulation of CK18 expression and increased proliferative activity. To additionally investigate this important aspect, we typed a subset of primary breast carcinomas ($n = 83$) for the proliferation-associated antigen Ki-67 and showed a statistically significant correlation to down-regulation of CK18 (data not shown). Moreover, our recent cell culture experiments on bone marrow micrometastases indicated that most of the proliferating tumor cells lack a detectable expression of the CK18 protein (27).

To additionally investigate the role of luminal CK18 in tumor progression, we performed a clinical follow-up analysis on the patients analyzed in our study. We could demonstrate that CK18 might play an important role as putative suppressor of breast cancer progression. Changes in the composition of the cytoskeleton of breast tumor cells may result in an increased plasticity, which is required for epithelial tumor cells to become mobile and invasive (28). This might explain why down-regulation of luminal CKs (*e.g.*, CK18) in primary tumors is correlated significantly with the onset of primary hematogeneous metastasis, as indicated by the presence of micrometastatic cells in bone marrow of node-negative breast cancer patients (14). Indeed, recent experimental data showed that manipulation of CK18 expression in cancer cells has considerable impact on their plasticity and motility (29). Apart from providing mechanical stability to epithelial cells, CK18 appears to restrict the motility of these cells. Most cancer cells do not have the physical properties of leukocytes constructed to invade tissues, circulate in the blood, and then re-enter new organs. To fulfill these requirements epithelial tumor cells need to undergo a fundamental phenotypical change called epithelial-to-mesenchymal transition. Epithelial-to-mesenchymal transition is an important developmental program, and epithelial tumor cells are able to reinitiate this program during their development toward more dedifferentiated and malignant states (28, 30). A reduced CK expression might among other factors lead to more dedifferentiated tumor cells with a higher mitotic rate, as this study revealed.

Besides down-regulation of luminal CKs constitutively expressed in normal breast cells, ectopic expression of CK5 and 7 typical for basal cells in stratified epithelia has been implicated in breast tumor progression (31–32). Moreover, breast tumor cells are even capable of ectopic expression of vimentin, the cytoskeletal intermediate filament typical for mesenchymal cells (7), and vimentin coexpression has been also suggested as a prognosticator in breast cancer (33). Both basal-like CKs and vimentin are mainly expressed in less differentiated breast tumors (7, 33, 34). Thus, it would be very interesting in future studies to explore the signaling pathways that lead to a concerted deregulation of intermediate filament expression.

The regulation of CKs occurs via several modalities, which might be responsible for the reduced CK expression observed in this study. Regulatory mechanisms include interaction with associated non-CK proteins (KAPs), resulting in CK phosphorylation, glycosylation, transglycosylation, caspase cleavage, ubiquitination, or association with other cytoplastic or cytoskeletal elements (6). The KAPs include adapter/signaling molecules such as 14-3-3 proteins, which are involved in the cell cycle regulation, tumor necrosis factor receptor type 2, and kinases such as protein kinase Cε-like and Jun kinases (p38), which carry out at least some of the phosphorylation events after stress induction (6).

In conclusion, our findings suggest that loss of CK18 expression might be critical to breast tumor progression. It is tempting to speculate whether CK18 plays an active role, or whether the observed changes at the expression level of CK18 merely reflects more upstream processes. In this context, the RAS signal transduction pathway might play a role, because the first intron of the *CK18* gene possesses an enhancer that directly interacts with the RAS-pathway-related transcription factors c-JUN and c-FOS via a conserved activator protein binding site, and low levels of c-JUN and c-FOS have been shown to suppress CK18 expression in murine carcinoma cells (35). The

better understanding of the biology driving metastatic spread opens the way for the development of more effective antimetastatic treatment strategies, such as pharmacological up-regulation of CK18.

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