Virchows Arch (1998) 433:119–129

ORIGINAL ARTICLE

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Biological and prognostic significance of stratified epithelial cytokeratins in infiltrating ductal breast carcinomas

Received: 26 August 1997 / Accepted: 11 February 1998

Abstract The biological significance of the differential expression of cytokeratin (CK) polypeptides in breast carcinomas is unclear. We examined the CK profiles of 101 primary infiltrating ductal breast carcinomas using monoclonal antibodies directed against 11 different CKs and against vimentin. Two major CK phenotypes were distinguished: first, a phenotype expressing only the simple-epithelial CKs 7 (variably), 8, 18 and 19, and secondly, a bimodal phenotype co-expressing significant amounts of one or more of the stratified-epithelial CKs 4, 14 and 17. The vast majority of G1 and G2 carcinomas had the simple-epithelium phenotype, as did a subgroup of G3 carcinomas. Interestingly, the majority (62%) of G3 carcinomas exhibited the bimodal phenotype, with the expression of CKs 4, 14 and 17 being statistically correlated with poor histological differentiation and absence of steroid hormone receptors. The distribution of vimentin only partially overlapped with that of these stratified-epithelial CKs. Prognostic analyses suggested that the presence of CKs 4, 14 and/or 17 was associated with short overall and disease-free survival in subgroups comprising G3, oestrogen-receptor-negative and vimentin-negative tumours. In node-positive tumours the correlation between these CKs and a shorter disease-free interval attained statistical significance (log rank, 0.0096). Thus, abnormal CK profiles in ductal breast carcinomas appear to reflect disturbed regulation of differentiation-related gene expression programmes and may prove to be of clinical value.

Key words Intermediate filaments · Cytokeratins · Vimentin · Prognosis · Breast cancer

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Introduction

As breast cancer is one of the most common causes of death in middle-aged women, much research has been devoted to finding prognostic markers that might facilitate the selection of individually adjusted therapeutic approaches. Among the most important predictive factors of proven worth are the involvement of regional lymph nodes, the histological type and grade, tumour size and steroid receptor status [1, 6, 8, 19]. Recently, prognostic value has been claimed for several molecular variables, including cell proliferation markers, oncogenes, proteolytic enzymes, adhesion molecules and receptor proteins (e.g., [1, 8, 9, 38]).

It has also been suggested that certain constituent proteins of the cytoskeletal intermediate filaments (IFs) may be of relevance with respect to the biological behaviour and prognosis of breast carcinomas [9, 12, 14, 26, 43, 46, 50, 52]. IF proteins comprise a complex multigene family embracing several different types (types I–VI), whose expression patterns are closely related to cell differentiation (for reviews, see [28–30, 34, 45]). The 20 human cytokeratins (CKs) that have been identified so far are classified as acidic type-I IFs (CKs 9–20) and neutral-basic type-II IFs (CKs 1–8) and are characteristic features of epithelial cells [11, 30, 33]. The type-III IF protein vimentin is typically present in mesenchymal

cells, but it may be co-expressed with CKs in certain epithelial cell types [3, 34].

Epithelial tissues exhibit characteristic patterns of CK expression that are principally governed by the cell type and cell differentiation, although they may also be modified by environmental conditions, proliferation activity and malignant transformation [11, 28–31, 34, 45]. With respect to the mammary gland, biochemical studies have shown that the lobular and ductal parts of normal human breast contain both simple-epithelium-type CKs (CKs 7, 8, 18, 19) and stratified-epithelium-type CKs (CKs 5, 14, 15, 17) [30, 31]. More recently immunohistochemistry has demonstrated that luminal cells express mainly the former type of CKs [2, 4, 17, 35, 42], with CK 19 showing some distinct heterogeneity [4] although some subpopulations of these cells (mainly in the ducts) additionally express certain stratified-epithelial CKs, such as CKs 5, 6, 13, 14, 15/16 and 17 [28, 35, 40]. In contrast, basal/myoepithelial cells almost exclusively express the latter CK type, comprising CKs 5, 6, 14, 15 and 17 [2, 7, 12, 15, 17, 21, 35, 58, 59], these CKs being most prominent in ductal myoepithelial cells. Myoepithelial cells also consistently express vimentin [3, 15] and sometimes contain glial fibrillary acidic protein (GFAP), another type-III IF protein [15].

Although epithelial tissues tend to retain their characteristic CK pattern during malignant transformation, modulations may occur within a certain range of possibilities during the development and progression of carcinomas [28–30, 34, 45]. Biochemical and immunohistochemical studies have shown that breast carcinomas usually retain the simple-epithelium CK pattern of normal luminal epithelial cells [2, 21, 30, 31]. However, several investigators have reported the additional presence of certain stratified-epithelial CKs as well as vimentin in some ductal breast cancers, as detected using biochemical [30, 31] and immunohistochemical procedures [2, 12, 15, 17, 35, 44, 51, 58, 59]. Unfortunately, these studies have often yielded conflicting data with respect to the distribution of individual CKs.

Several studies dealing with the co-expression of vimentin in infiltrating ductal carcinomas have indicated that the presence of this IF protein is correlated with poor histological differentiation and the loss of steroid hormone receptors [9, 14, 43, 47]. In the case of CKs, only one prospective clinical study concerned with CK-14-positive breast carcinomas has given grounds for the supposition that a poorer prognosis must be expected in cases expressing this stratified-epithelial CK [12].

With an extended panel of CK antibodies applied to frozen tumour samples obtained from a large number of cases, the present study aimed to establish precise CK profiles of infiltrating ductal carcinomas of the breast and to examine whether these findings were correlated with various clinical and pathological parameters. Particular attention was given to the possible importance and prognostic relevance of the additional expression of stratified-epithelium-type CKs as against the co-expression of vimentin.

Materials and methods

Between 1986 and 1992, we collected a total of 101 primary infiltrating ductal breast carcinomas not otherwise specified (NOS) at the Institute of Pathology and the Department of Gynaecology of the University of Mainz, Germany. Immediately after surgery, the tissue specimens were frozen in liquid-nitrogen-cooled isopentane and stored at –75°C. Parallel paraffin-embedded sections were routinely examined to obtain information about the histological type and grade [6] of the tumour (10 G1, 46 G2 and 45 G3 carcinomas) and about lymph-node involvement (38 pN0, 33 pN1, 4 pN2 and 25 pNx [20]). In 90 cases, the oestrogen receptor (ER) and progesterone receptor (PR) levels were evaluated biochemically by applying the dextran-coated-charcoal (DCC) method. An immunocytochemical assay (ER-ICA, Abbott Laboratories, North Chicago, Ill.) was applied to frozen sections from 37 cases (including those 11 cases not subjected to biochemical analysis). The results were comparable when cut-off levels of 20 fmol/mg cytosolic protein (DCC) and 10% stained tumour cell nuclei (ER-ICA) were used.

In all cases information was available concerning the patient's age, the tumour size (pT) and the presence or absence of nodal metastases (pN), according to the TNM classification [20]. In 51 cases, we had access to further data relating to the occurrence of distant metastases (M), local recurrence and survival, with the follow-up interval ranging from 10 to 73 months (mean follow-up, 36

Table 1 Monoclonal antibodies (*MAbs*) used for immunohistochemical detection of cytokeratin polypeptides in infiltrating ductal breast carcinomas

MAb	Specificity	Source	References
6B10	CK 4	Euro-Diagnostica, Apeldoorn, The Netherlands	see [28]
AE14	CK 5	Prof. T.-T. Sun, New York University Medical Center, New York, USA	see [28]
CK-7	CK 7	Boehringer Mannheim, Mannheim, Germany	see [28]
CAM5.2	CK 8	Becton-Dickinson, Heidelberg, Germany	see [28]
2D7	CK 13	Euro-Diagnostica, Apeldoorn, The Netherlands	see [28]
Ks8.12	CK 13, 15, 16	BioMakor, Rehovot, Israel	see [28]
LL001	CK 14	Dr. E. B. Lane, University of Dundee, Dundee, UK	[41]
Ks17.E3 (E3)	CK 17	Progen, Heidelberg, Germany	[17]
SK2-27	CK 14,17	Dr. P. Leoncini, Sclavo Research Center, Siena, Italy	[10]
Ks18.174	CK 18	Prof. W.W. Franke, German Cancer Research Center, Heidelberg, Germany	see [28]
Ks19.2 (Z105.6)	CK 19	Progen, Heidelberg, Germany	see [28]
IT-Ks20.3	CK 20	Progen, Heidelberg, Germany	[33]
IT-Ks20.5	CK 20	Progen, Heidelberg, Germany	[33]
IT-Ks20.10	CK 20	Progen, Heidelberg, Germany	[33]
V9	Vimentin	Sanbio, Uden, The Netherlands	see [43]

months; median follow-up, 39 months). Most of these 51 patients were treated with partial resection of the breast and after surgery underwent radiotherapy with a total dosis of 50 Gy when the tumour size did not exceed 3 cm. In most cases with tumour size >3 cm a modified radical mastectomy [39] was carried out. Regardless of the type of breast surgery, radical axillary node dissection including level III was additionally performed.

For immunohistochemistry, frozen sections (thickness, 4 μm) were dried at room temperature for 12 h before being fixed in acetone for 10 min at –20°C. Immunohistochemical staining was performed using the indirect immunoperoxidase technique, with 3,3-diaminobenzidine-tetrahydrochloride serving as the chromogenic agent [32, 33]. The specificities and sources of the employed monoclonal antibodies, together with relevant literature references, are listed in Table 1. Frozen sections of each carcinoma were also examined using H&E staining compared with the routine paraffin sections, the aim being to ensure that the frozen tissue block used was a truly representative sample of the case under consideration.

The labelling results were classified on the basis of the estimated percentage of positive tumour cells, irrespective of the staining intensity, and divided into the following catagories: diffusely positive (>80% positive tumour cells); heterogeneously positive (21–80% positive tumour cells); focally positive (6–20% positive tumour cells); negative (0–5% positive tumour cells). Although completely negative immunoreactions and single cell staining in up to 5% of tumour cells were recorded separately, these two groups were subsequently merged and regarded as negative (see below). Photomicrographs were taken using a Leitz Diaplan microscope and Agfapan 25 film.

Data were statistically processed using the SAS-PC programme (version 6.03; SAS, Cary, N.C.). Correlations between different CKs or vimentin expressions were evaluated by analysis of variance. Chi-square tests with contingency tables were applied to analyse the correlation between the distributions of CKs and vimentin among different groups, as defined by grading, steroid hormone receptors, local recurrence, distant metastases and patient's age. The disease-free interval and overall survival were computed according to the method of Kaplan and Meier [23]. Chi-square values were calculated by log-rank statistics [22]. A P-value of <0.001 was taken to indicate a very significant correlation, while a value of $P<0.05$ was considered to reflect a good significant correspondence. All tests were performed using both a >0% and a >5% threshold of immunohistochemical staining positivity. There were no significant differences between the statistical results obtained for the two thresholds, suggesting that instances of very focal CK expression and the presence of single positive cells (<6%) are of little biological significance. Therefore we used the 5% cut-off level for the final evaluations.

Results

Tumour specimens of 101 cases of primary infiltrating ductal breast carcinoma were analysed using immunohistochemistry to ascertain the distribution of various IF proteins. Table 2 summarizes the immunostaining results obtained for each CK; a typical example of a G2 carcinoma is illustrated in Fig. 1a–c, while the staining patterns observed in various G3 carcinomas are shown in Figs. 1d–j and 2a–m. Nearly all the carcinomas examined were strongly stained by MAbs directed against the simple-epithelial CKs, 8, 18 and 19 (Fig. 1a, e, h; Fig. 2b). The immunostaining for CK 7 varied considerably; indeed, 9 of the 101 cases were entirely negative, whereas the majority of tumours exhibited heterogeneous (46 cases) or diffuse staining (34 cases; Fig. 1b). The presence and level of CK-7 expression were well correlated with G3 tumours (P=0.038) and with negative ER status (P=0.001).

An interesting finding was the detection of significant heterogeneous or even diffuse staining for CK 20 in three G3 carcinomas (Fig. 1i, j). The specificity of this CK-20 staining was confirmed by the use of three different anti-CK-20 MAbs. It is noteworthy that these cases shared some similarity, in that all of them were negative for steroid hormone receptors and were composed histologically of small rounded, solid anastomosing formations of relatively large tumour cells (Fig. 1g). However, these cases differed from each other in focal expression of CK 4 or CK 14/17 (see below) in 1 case each.

A surprisingly high number of cases were also stained by MAbs directed against the stratified-epithelial CKs 5, 4, 13, 13/15/16, 14 and 17, as well as against vimentin. The distribution of these CKs showed considerable variation, this appearing to be correlated with the grade of tumour differentiation. Therefore, the results obtained for each tumour grade are presented separately in Table 2.

As to the group of G1 and G2 carcinomas (together 56 cases), about one-fifth (12 cases) yielded positive staining for MAb Ks8.12, which detects CKs 13, 15 and 16. This staining probably reflects the presence of CK 15 and/or CK 16, since CK 13 was less frequently detected

Table 2 Expression of CK polypeptides and vimentin in different histological grades of infiltrating ductal breast carcinomas

Cytokeratin polypeptides	Grade 1			Grade 2			Grade 3		
	n[a]	Positive cases[b]	[%]	n	Positive cases	[%]	n	Positive cases	[%]
CK 18	5	5	100	25	25	100	18	18	100
CK 19	10	10	100	46	44	96	45	44	98
CK 7	10	10	100	46	37	80	45	43	96
CK 20	10	0	0	46	0	0	45	3	7
CK 13	9	1	11	31	2	6	41	0	0
CK 13/15/16	10	4	40	46	8	17	45	15	33
CK 4	10	2	20	46	1	2	45	16	36
CK 5	10	1	10	46	4	9	45	8	18
CK 14	10	0	0	46	1	2	45	9	20
CK 17	10	1	10	46	2	4	45	17	38
CK 14/17	10	1	10	46	2	4	44	22	50
Vimentin	10	2	20	46	3	7	45	21	47

[a] Number of cases examined
[b] Positive cases as defined by staining of more than 5% of tumour cells

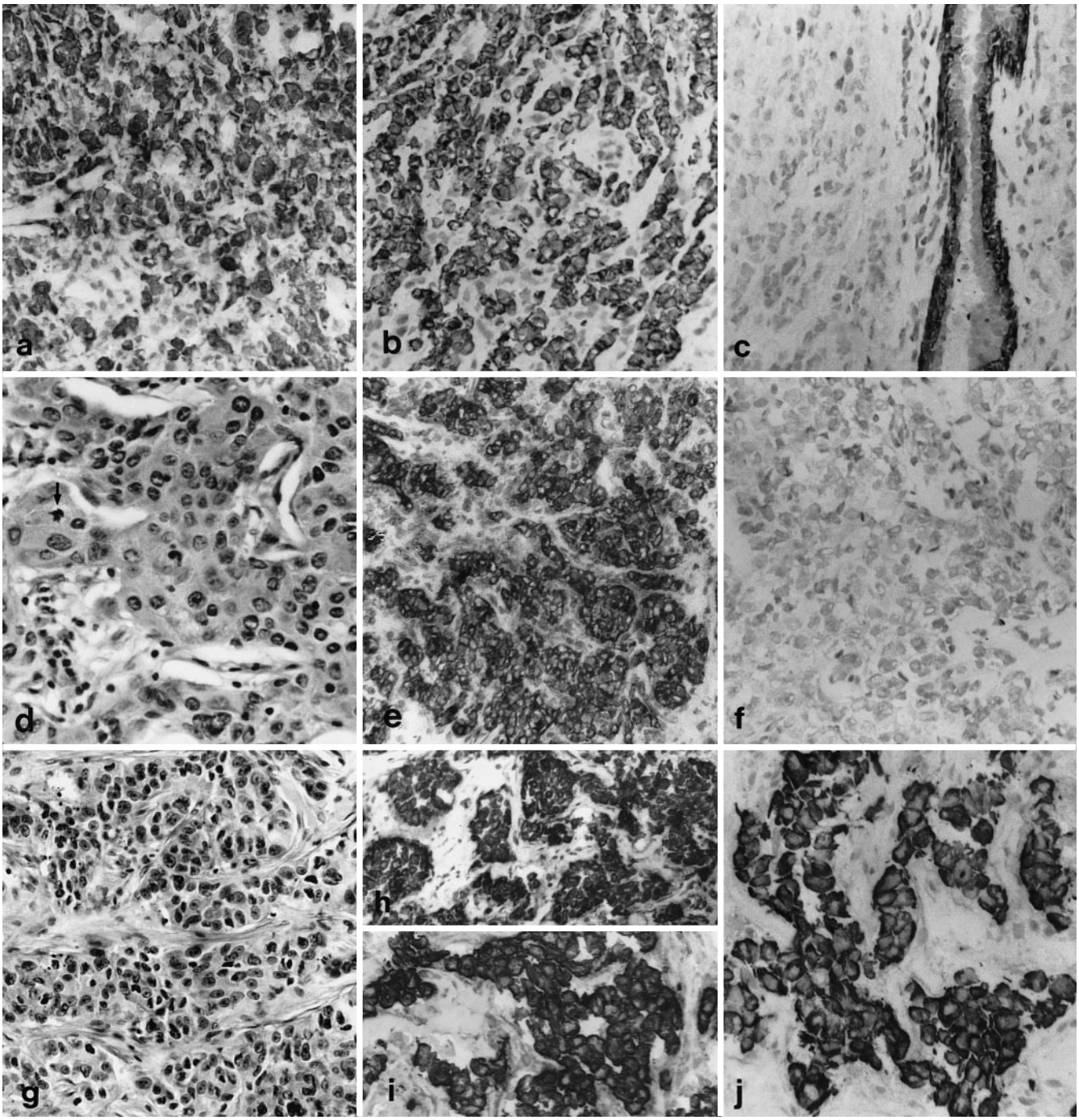

Fig. 1a–j Breast cancers lacking the expression of stratified-epithelial CKs. **a–c** Immunohistochemical staining of frozen sections of a G2 infiltrating ductal breast carcinoma not otherwise specified (NOS), exhibiting the simple-epithelial CK pattern: there is diffuse immunohistochemical staining for **a** CK 19 and **b** CK 7. **c** Monoclonal antibody SK2–27, directed against CKs 14 and 17, clearly stains the basal myoepithelial layer of a normal duct; however, there is no staining of tumour cells. **d–f** G3 breast carcinoma NOS showing the CK pattern of the simple-epithelium phenotype: **d** H&E staining (paraffin-embedded tissue): note the solid growth pattern with apparent nuclear and cell polymorphism (*arrow* denotes mitosis). **e** Immunohistochemical staining for CK 19 produces a positive reaction in more than 80% of the tumour cells. **f** No staining of tumour cells for CKs 14/17 is visible. **g–k** CK-20-positive G3 carcinoma growing in rounded cell clusters as shown in the H&E stained paraffin section (**g**), showing diffuse immunohistochemical staining not only for CK 19 (**h**) but also for CK 20, as recognized by MAbs IT-Ks20.5 (**i**) and IT-Ks20.3 (**j**). **a–c**, **f**, **g**, **i**×180; **d**×280; **e**×120, **h**×110, **j**×225

Table 3 Typing of grade G3 infiltrating ductal breast carcinomas according to their expression of the stratified-epithelial CKs 4, 14 and 17 [– negative (including single positive cells up to 5%), + 6–20% positive cells (focal staining), ++ 21–80% positive cells (heterogeneous staining), +++ >80% positive cells (diffuse staining), *ER* oestrogen receptor, *PgR* progesterone receptor]

Type	Subtype	*n*	CK4	CK5	CK13[a]	CK13/15/16	CK14	CK17	CK14/17	CK7	CK19	CK20	Vimentin	ER	PgR[a]
Simple epithelium phenotype		17	–17	–17	–16	–14 +1 ++2	–17	–17	–16 +1	–2 +1 ++10 +++4	–1 ++1 +++15	–15 ++1 +++1	–15 +2	Neg. 12 Pos. 5	Neg.12 Pos. 3
Bimodal phenotype	CK-4 subtype	8	+4 ++4	–8	–7	–7 +1	–8	–8	–6 +1[a]	++3 +++5	+++8	++1	–8	Neg. 7 Pos. 1	Neg. 7
	CK-4/17 subtype	7	+1 ++6	–5 ++2	–5	–5 +1 ++1	–7	+1 ++6	+2 ++5	++2 +++5	+++7	–7	–1 +1 ++5	Neg. 7	Neg. 4 Pos. 1
	CK-14/17 subtype	13	–12 +1	–7 +3 ++3	–11	–4 +5 ++4	–4 +3 ++5 +++1	–3 +2 ++6 +++2	+1 ++7 +++5	+1 ++6 +++6	+++13	–13	+3 ++8 +++2	Neg. 12[a]	Neg. 11

[a] Not tested in all cases.

alone by a specific MAb. The majority of G1 and G2 tumours were negative for CKs 4, 5, 14 and 17 (Fig. 1c). Only 6 of the 56 cases revealed mostly focal or heterogeneous staining for one or more of CKs 4, 14, and 17. Another 4 cases showed focal or heterogeneous staining with the antibody against CK 5. Focal or heterogeneous co-expression of vimentin was detectable in 5 cases only. Most of the G1 and G2 carcinomas yielded positive findings for both ERs (86%) and PRs (73%).

Interestingly, among the 45 G3 carcinomas examined, the additional expression of stratified-epithelial CKs was much more frequently observed: 16 of these carcinomas were heterogeneously positive for CK 4 (Fig. 2c, e), while 9 showed prevailingly heterogeneous staining for CK 14 (Fig. 2f, l). Also, 15 cases were mostly heterogeneously, and 2 cases, diffusely immunostained by antibody E3 directed against CK 17 (Fig. 2g, k); a similar staining pattern was produced by antibody SK2–27, which also recognizes CK 14 (Fig. 2j). The type-II partner of this last CK, i. e. CK 5, was found in 8 of the cases, while immunostaining for CK 13/15/16 was observed at higher frequency, i.e. in 15 cases. Co-expression of vimentin - often heterogeneously and sometimes diffusely immunostained - was seen in nearly half the G3 carcinomas, i.e. 21 cases (Fig. 2h, m).

Two major phenotypes of ductal breast carcinomas were distinguishable on the basis of the following staining patterns, in particular by the presence or absence of the stratified-epithelial CKs 4, 14 and/or 17. These CKs were rarely detected in G1 and G2 carcinomas (6/56 cases). However, they were much more frequently expressed in G3 carcinomas (27/45 cases). Therefore, our further analyses of these phenotypes first concentrate on G3 carcinomas. We defined the phenotypes as follows (Table 3):

Simple-epithelium phenotype. These G3 carcinomas (17 out of 45 cases; 38%) were found to express the simple-epithelial CKs 7 (variably), 8, 18 and (mostly) 19 (Fig. 1d–f), but lacked significant (above the 5% threshold) expression of CKs 4, 14 and 17 (the reactivity with MAb Ks8.12 being ignored for the present purposes). Only 2 cases exhibited the focal co-expression of vimentin. Two cases expressed CK 20 (see above). The majority of these G3 cases were receptor negative, but with 5 cases exhibiting ERs and 3 cases showing PRs. [Among G1 and G2 carcinomas, 50/56 cases (89%) could be assigned to the simple-epithelium phenotype.]

Bimodal phenotype. These tumours expressed both the simple-epithelial CKs along with one or more of the stratified-epithelial CKs 4, 14 and 17. This phenotype accounted for 28 of the 45 G3 carcinomas examined (62%), and it was possible to subdivide this group into three subtypes. The *CK-4 subtype* (8 cases) was characterized by the focal or heterogeneous expression of CK 4, with CKs 14 and 17 being essentially absent (Fig. 2a–c); 1 case expressed CK 20 (see above). Vimentin was undetectable, while most of these cases were devoid of hormone receptors. The *CK-4/17 subtype* (7 cases) exhibited pronounced expression of CKs 4 and 17,

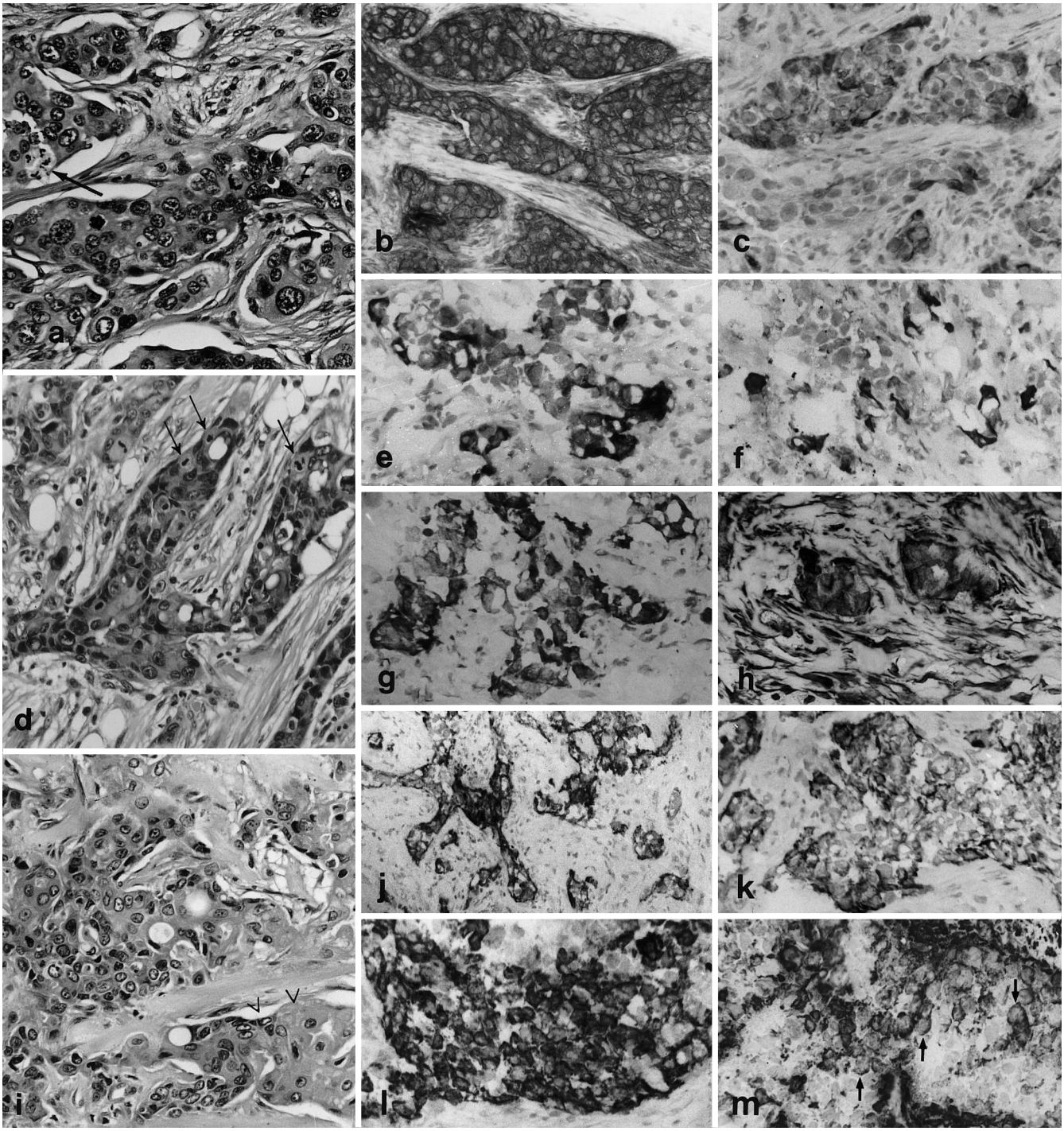

Fig. 2a–m G3 breast cancers showing expression of stratified-epithelial CKs (bimodal phenotype). **a–c** G3 breast carcinoma NOS corresponding to the CK-4 subtype: **a** H&E staining reveals irregular, solid trabecular growth with marked nuclear and cell polymorphism and pathologic mitotic figures (*arrow*). **b** There is diffuse immunohistochemical staining of all tumour cells for CK 19. **c** Detection of CK-4 staining of variable intensity in approximately 40% of tumour cells. **d–h** G3 breast carcinomas NOS falling into the CK-4/17 subtype: **d** H&E staining shows a trabecular growth pattern and stromal desmoplasia; note the marked polymorphism and numerous mitoses (*arrows*). **e** Immunohistochemical staining for CK 4 with faint colouring of about 60% of the tumour cells. **f**, **g** While there are just a few scattered CK-14-positive tumour cells (**f**) there is a strong reaction in more than 60% of the tumour cells for CK 17 (**g**). **h** There are small tumour cell clusters positive for vimentin; also note the staining of the surrounding stromal cells. **i–m** G3 breast carcinoma NOS of the CK-14/17 subtype: **i** H&E staining reveals an irregular solid growth pattern with focal changes suggestive of squamous metaplasia (e.g. *arrowheads*), marked pleomorphism and focal necrosis. There is remarkably diffuse immunohistochemical staining for CK 14/17 (**j**) and CK 14 alone (**l**), while the staining for CK 17 alone (**k**) reveals a somewhat more heterogeneous pattern. **m** There are numerous vimentin-positive tumour cells (*arrows*) next to strongly positive stromal cells. **a**×280; **c**, **d**, **g–i**, **k–m**×180; **e**×200; **b**, **f**, **j**×110

while CK 14 was either totally absent or was merely encountered in a few scattered individual cells (Fig. 2d–h). Nearly all of these cases co-expressed vimentin, and 2 cases were positive for CK 5. The *CK-14/17 subtype* (13 cases) was defined on the basis of the expression of CK 14 and/or CK 17 (usually both) along with the absence of CK 4 (Fig. 2i–m); all cases again expressed vimentin, while many also expressed CK 5. The vast majority of the G3 carcinomas of this bimodal phenotype were entirely devoid of steroid receptors. [In G1 and G2 carcinomas, the bimodal phenotype was noted in only 6 out of the 56 cases studied (11%).]

In order to ascertain whether the expression of stratified-epithelial CKs in ductal breast carcinomas might be of prognostic significance, statistical analyses were performed. Employing chi-square testing, the recorded expression patterns of CKs 4 and 17, and of vimentin, were each very well correlated with a poor degree (G3) of differentiation (all $P<0.001$), with a particularly good correlation being observed for CK 14 ($P=0.002$). Similarly, there was a close correlation between each of these four IF proteins and a negative status for ERs (all $P<0.001$). As expected, the absence of steroid hormone receptors was highly correlated with a low level of histological differentiation (ER and PR: $P<0.001$). No statistically demonstrable correlations with grading were observed for CK 5 or for CK 13/15/16. In addition, the expression of each of CKs 14 and 17 was well correlated with vimentin co-expression.

To recognize statistical correlations both between the presence of CKs 4, 14 and 17 (as a group) and other prognostic variables and for follow-up studies, the tumour group that exhibited the bimodal phenotype was compared with the group showing the simple-epithelium phenotype. Chi-square testing revealed no correlation between patient age, local recurrence, tumour size or nodal status and the expression of CKs 4, 14 or 17, although a tendency emerged suggesting the more pronounced expression of CKs 4 and 17 (but not CK 14) in larger tumours (>pT1). While the bimodal phenotype appeared to be more frequent in cases with distant metastases, this finding failed to attain statistical significance. However, hormone-receptor negativity was closely correlated with stratified-epithelial-CK positivity, i.e., the bimodal phenotype (ER, $P<0.001$; PR, $P=0.008$).

The 51 cases for whom follow-up data were available were correlated with the pathological and immunohistochemical data obtained. Kaplan-Meier analyses demonstrated that patients with low-grade (G1) and ER-positive carcinomas had longer disease-free intervals and more favourable overall survival rates than tumours of higher grades (G2 and G3) or ER-negative tumours; however, the findings for these 51 cases did not attain statistical significance.

The disease-free interval was found to be shorter in bimodal cases, although this correlation again did not reach the level of statistical significance (log rank=0.061; Fig. 3). A similar, albeit weaker, tendency emerged with respect to the overall survival rate, with 66.9% of patients with bimodal-type tumours being alive at the end of the follow-up interval of 73 months, compared with 81.3% of the patients with simple-epithelium-type tumours. Similar trends were found in the expression of vimentin. Both disease-free interval and overall survival rate were more favourable in vimentin-negative cases (65.4% and 80.8%, respectively) than in vimentin-positive tumours (54.4% and 68.4%).

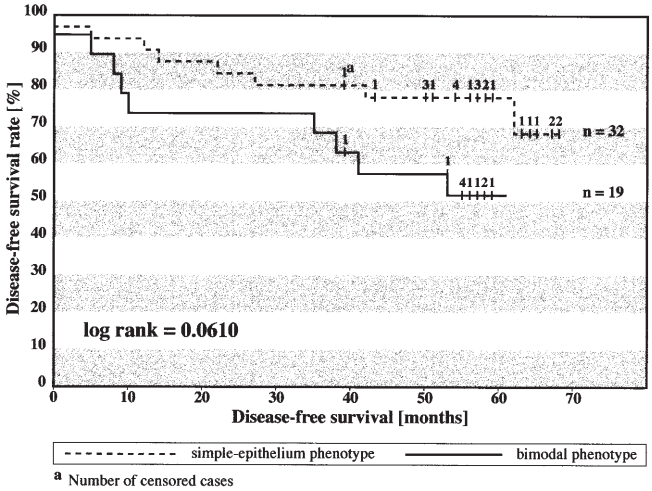

Fig. 3 Disease-free survival rates concerning the expression of the stratified-epithelial CKs 4, 14 and/or 17 (bimodal phenotype) in 51 unselected patients with infiltrating ductal breast carcinomas NOS. [a]No. of censored cases

To test whether the expression of CKs 4, 14 and/ or 17 might be able to provide useful prognostic information independent of established prognostic parameters, we applied Kaplan-Meier analysis to subgroups in which the findings were homogeneous for the prognostic factors (tumour grading, steroid hormone-receptor status, node status and vimentin expression). For the prognostically favourable subgroups (grades G1/G2, ER positive, node negative), there were no significant differences between cases with negative and positive findings for stratified-epithelial CKs; however, it should be pointed out that these subgroups were rather small and probably not representative. In contrast, in the subgroup of vimentin-negative cases, patients with bimodal-type tumours exhibited a less favourable tendency with respect to overall survival (60.0% versus 84.6% for simple-epithelium-type tumours; not significant) and shorter disease-free intervals, this last finding almost attaining statistical significance (log rank=0.062). For patient subgroups with an unfavourable prognosis (G3, ER negative, node positive), less favourable disease-free and overall survival rates emerged for bimodal-type tumours (not significant for G3 and ER negative). Very notably, however, statistical significance (log rank=0.0096) was obtained for node-positive tumours (Fig. 4): the disease-free interval was found to be significantly shorter in patients with bimodal-type tumours than in those with simple-epithelium-type tumours lacking these CK proteins.

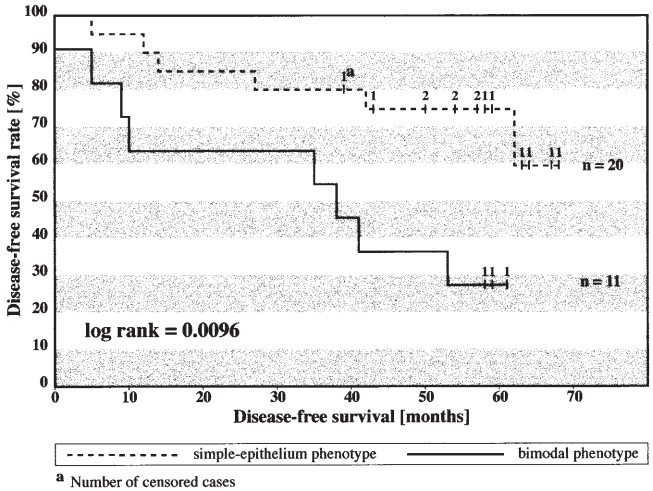

Fig. 4 Disease-free survival rates concerning the expression of the stratified-epithelial CKs 4, 14 and/or 17 (bimodal phenotype) in a subgroup of 31 patients with infiltrating ductal breast carcinomas NOS with nodal metastases. [a]No. of censored cases

Discussion

For almost two decades, the expression of CKs in breast carcinomas has been the subject of intensive research efforts. Even early studies yielded findings – whose essential validity has been confirmed in the present study – indicating that the simple-epithelial components, CK 8, CK 18, CK 19 and, more variably, CK 7, are prevalently expressed in such tumours [2, 21, 30, 31, 35–37, 42, 44], thereby suggesting that the luminal-cell phenotype is predominant. Normal luminal cells, though, do exhibit diffuse and intense CK-7 expression [2, 28, 42]. In accordance with the present findings, Nathrath and Lane [36, 37] have reported a positive correlation between the level of CK-7 expression and both an elevated degree of malignancy and the absence of ER; these investigators also found the recurrence rate to be higher for strongly CK-7-positive breast cancers than for tumours exhibiting little or no staining for this CK. Recent reports have suggested that the mere presence, or a high intensity, of immunostaining for the simple-epithelial components, CK 8 [50] and CK 18 [46], might be a favourable prognostic parameter. Such findings of the heterogeneous expression of these primary simple-epithelial CKs – which stand in striking contrast to the mostly homogeneous diffuse staining (thus precluding their use for prognostic assessment) observed by ourselves and many other investigators - may in fact be attributable to the different techniques applied (fixation, antibodies, staining method) and/or the occurrence of epitope masking. Another simple-epithelial component, CK 20, was found to be absent in most breast carcinomas, which is in agreement with the results of earlier studies ([24, 33]; see [29] for further references); indeed, the lack of this CK is considered to be a criterion for ruling out the possibility that a tumour under investigation might be a metastatic adenocarcinoma, as such tumours often express this gastrointestinal CK [24, 29, 33]. What would appear, at first glance, to be an exceptionally high proportion (19%) of a group of breast carcinomas being reported as CK-20 positive by Wang et al. [56] was due to their positive assessment of tumours containing scattered cells that exhibited often granular, equivocal CK-20 staining. In our series, a very small subset of G3 carcinomas (3 cases) exhibited widespread immunostaining that could be attributed unequivocally to the presence of CK 20. Despite certain similarities, these cases were heterogeneous with respect to the focal presence of CK 4 and CK 14/17 (detected in 1 case each). Thus, it would be premature as yet to propose that such tumours might comprise a distinct subtype of breast carcinomas.

In early biochemical studies, we reported that a minor subgroup of ductal breast carcinomas contain small amounts of stratified-epithelial CKs, including the basal-cell-type CK 14 and CK 17 [30, 31]. In subsequent immunohistochemical studies, including the present one, these findings have been confirmed and extended to the cellular level. The most widespread stratified-epithelial CK reactivity is elicited by MAb Ks8.12, producing focal staining in 27% of our tumour cases, a result that is in close agreement with published data [40, 54, 59], although in another study the majority of cases was positive [44]. The staining specificities of MAb Ks8.12 appear to differ considerably from those of the MAbs against the other stratified-epithelial CKs, since Ks8.12 positivity is seen in a considerable subset of normal luminal epithelial cells but not in myoepithelial cells and, in breast carcinomas, does not show a strong preference for G3 tumours (Table 2; cf. [44]). CK 16, one likely antigen recognized by MAb Ks8.12 in normal and malignant breast tissue (see “Results”), is known to be a marker of hyperproliferation in stratified squamous epithelia [57]. In breast carcinomas, however, the low degree of concurrence between the distribution of CK 16 or CK 13/15/16 and that of the proliferation marker, Ki 67, is noteworthy [59].

The present finding of the preferential occurrence of focal CK-5 expression in high-grade breast carcinomas is at variance with the proposal of Trask et al. [53], based on cell-culture studies, that CK 5 is a marker of normal, as opposed to malignant, breast epithelium. There is preliminary evidence that CK 6, which is related to CK 5, is also expressed predominantly in high-grade breast cancers (U. Hesse, R.B. Nagle, and R. Moll, unpublished results).

The stratified-epithelial CK whose expression has been most frequently reported in a subgroup of breast carcinomas is CK 14. Our detection of CK 14 in 10% of the cases examined is comparable with a number of previously published results [2, 12, 15, 21, 35, 58, 59]; some investigators, though, have reported the incidence of this CK in such tumours to be either much lower [36, 37, 54] or much higher [44], this divergence possibly being due to the application of different CK-14 antibodies and/or staining methods. While Su et al. [49] were able to detect CK-14 mRNA in 3 of a group of 12 breast carcinomas, CK-14 protein proved to be present in only 2 of these tu-

mours, pointing to the occurrence of certain disturbances in expression regulation. The incidence of CK 17, which was detected in 21% of the cases examined in the present study (for a similar value, see [44]), therefore appears to be higher than that of CK 14; in other studies, CK 17 was found to be present in about one-third of the cases investigated [17, 59]. Another stratified-epithelial component that is mainly, but not exclusively, expressed in (non-cornifying) stratified squamous epithelia, CK 4, has yet to be detected in normal breast epithelium. Neo-expression of this CK was detectable in one-fifth of our cases, an incidence comparable to that reported by Heatley et al. [18]; in contrast, other investigators have found CK-4 expression to be a rare occurrence [37] or have failed to detect this CK in any of their series of breast tumours [44].

The biological significance of the expression of the stratified-epithelial components, CK 4, CK 14, and CK 17 (bimodal phenotype), in (for the most part) poorly differentiated breast carcinomas remains an enigma. One hypothesis is that CK-14 and CK-17 expression might indicate derivation from, or differentiation toward, myoepithelial cells. In fact, the expression of a-smooth-muscle actin has been reported in breast cancer cells [44, 48], its presence being detected by Santini et al. [44] in a considerable proportion of cases, most of which were of nuclear grade 3. However, in several other studies [7, 15, 27, 55], both this actin isoform and another myoepithelial marker, GFAP, have essentially eluded detection in the tumour cells of breast carcinomas. Furthermore, unlike tumours of the bimodal phenotype, normal myoepithelial cells do not co-express simple-epithelial CKs. Another explanation that has been suggested is that breast carcinomas expressing stratified-epithelial CKs acquire a more general basal-cell phenotype, characterized by the synthesis of certain basement-membrane proteins [58, 59]. A further possibility is that carcinomas of the bimodal phenotype develop certain differentiation features of a particular, minor, “bimodal” luminal cell type that is characterized by the expression of CK 14 and CK 17 (for references, see the “Introduction”), although this would not necessarily indicate their derivation from such cells. Böcker et al. [7] have suggested that a population of CK-5/14-positive normal luminal cells are “post-stem cells” and have reported the presence of tumour cells exhibiting similar CK expression patterns in a small proportion of ductal in situ carcinomas. It remains unclear whether infiltrating carcinomas of the bimodal phenotype are in some way related to such cells. The expression of stratified-epithelial CKs in certain breast carcinomas may also point to rudimentary squamous metaplastic processes, but then again, fully developed squamous metaplasia is rare in cases of breast cancer. From a more general point of view, the correlation of the more complex bimodal CK phenotype with a poor degree of differentiation may reflect those principles underlying the increased CK complexity observed in other types of carcinomas in the event of a decrease in the level of differentiation [28, 29]. Such complex neo-expression may stem from the inactivation of repressive regulatory factors [25].

In the present study, the bimodal phenotype was found to be significantly correlated with a high grade of malignancy, the absence of steroid hormone receptors, and vimentin co-expression. These results suggest that breast carcinomas of the bimodal phenotype may have a more aggressive biological course. Published studies supporting such a concept are by no means abundant. Dairkee et al. [12] have suggested that CK-14-positive breast carcinomas may have an unfavourable prognosis, while more recently Santini et al. [44] have defined a group of high-grade invasive breast carcinomas (“group 3”) comparable to the carcinomas of the bimodal phenotype delineated in the present study (although they were unable to detect CK 4 in their group). Santini et al. [44] also observed a positive correlation between this high-grade tumour “group 3” and an elevated proliferation index (Ki-67 score), a factor that is of immediate relevance for the biological behaviour of tumours; unfortunately, prognostic data were not included within the scope of their investigation. In another recent study of invasive breast carcinomas, Takei et al. [51] observed that the incidence (21%) of “high-molecular-weight-CK” positivity (as defined by MAb 34BE12) was positively correlated with the grade of malignancy and inversely correlated with ER status; however, these factors were not shown to have any significant influence on overall and relapse-free survival. Other investigators have reported much higher levels of 34BE12 staining in breast carcinomas [5, 16, 48] than those observed in the aforementioned study [51]. As the precise CK reactivity of this MAb remains uncertain, it is, at this point, hardly worthwhile attempting to compare the results of such studies employing MAb 34BE12 with those of either the present study or of another study involving a different MAb directed against high-molecular-weight CKs, which stained most of the human breast carcinomas investigated [27].

A larger body of prognostic data is available on the co-expression of vimentin in breast carcinomas. These indicate that this factor is correlated with a high grade of malignancy, negative ER status, a high growth fraction, and a poor overall survival rate [9, 14, 26, 43] (present findings), although a recent broad-based (albeit retrospective) study has concluded that vimentin expression is in itself not genuinely correlated with a poor prognosis [47]. In the present study, we were further able to demonstrate a close correlation between the co-expression of vimentin and the expression patterns of CK 14 and CK 17, while no such correlation emerged for CK 4. Whereas the CK-4 subtype therefore appears to differ in some respects from the other subtypes of the bimodal phenotype, we did find the expression of this CK also to be correlated with classical prognostic parameters (grading, ER status), a result not previously reported in studies by other investigators [18, 37].

With respect to the prognostic relevance of new variables such as CK expression, clearer insights are obtainable by applying Kaplan-Meier analyses than can be achieved by attempting mutual correlations of different prognostic variables. To our knowledge, this is the first

study dealing with CK expression in breast carcinomas in which Kaplan-Meier analyses have been performed. These analyses revealed a less favourable disease course for tumours with the bimodal phenotype than for those with the simple-epithelium phenotype. Moreover, when we analysed subgroups defined by other prognostic parameters, differences were revealed in the disease-free interval for prognostically unfavourable subgroups, and these proved to be of marked statistical significance (log rank=0.0096) for the subgroup of lymph-node-positive patients (Fig. 4). This opens up the possibility of further subtyping those breast carcinomas whose primary prognostic indicators suggest a less favourable course.

We have shown that, along with CK 14 which has already been suspected of having prognostic value [12] the occurrence of CK 4 and CK 17 is not only correlated with some of the major prognostic variables in breast carcinoma but also seems to have an unfavourable influence on both disease-free and overall survival rates, especially in high-risk patients. As to whether one or more of the polypeptides, CK 4, CK 14, and CK 17, might have any prognostic relevance for the nodal status, N0, or whether these molecules might be able to serve as independent prognostic parameters, still remains to be elucidated. Future multivariate analyses involving larger numbers of cases and then prospective clinical studies will be needed before such questions can be satisfactorily answered. The recent possibility of using advanced immunohistochemical techniques such as microwave enhancement [cf. 13, 47, 56] to detect most CKs in formalin-fixed, paraffin-embedded "routine" tissue should greatly facilitate the performance of such studies.

Acknowledgements The authors wish to thank Prof. Dr. K. Pollow (Department of Experimental Endocrinology, University of Mainz) for providing data on the steroid hormone receptors and Dr. E. Scheidt (Institute of Medical Statistics and Documentation, University of Mainz) for counselling on statistical problems. We also acknowledge A. Holzbach, U. Hesse, R. Baumann and P. Pulkowski for technical assistance and A. Cowin for his critical comments on the English.

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