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Identification of a subset of breast carcinomas characterized by expression of cytokeratin 15: Relationship between CK15+ progenitor/amplified cells and pre-malignant lesions and invasive disease

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ABSTRACT

Recently, we presented evidence – based on the analysis of benign hyperproliferative lesions of the breast – for the presence of cells that express the stem cell marker cytokeratin (CK) 15 in combination with CK19, a protein widely expressed by mammary epithelial cells. Here we report the finding of a subset of breast carcinomas characterized by expression of CK15. CK15 expressing tumors constituted 5% (6 out of 120; 4 of ductal type and 2 of lobular type) of the high-risk breast carcinomas examined by gel-based proteomics and immunohistochemistry. Five out of the six CK15+ carcinomas were CK15+/CK19−. The remaining tumor was mainly composed of cells expressing both CK15 and CK19 (CK15+/CK19+), but it also contained invasive areas with cells expressing only one of these makers (CK15+/CK19− and CK15−/CK19+ cells). To address the relationship between putative luminal progenitor/amplified CK15+ cells and malignant disease, and to determine whether cells/lesions lose expression of CK15 as a result of tumour initiation and/or progression, we searched among our sample set for carcinomas in which invasive tumor areas co-existed with non-malignant cells and hyperproliferative and known pre-malignant lesions. Only one such tumour was found (T71), a CK15−/CK19+/p53+ carcinoma that contained p53 negative non-malignant epithelial cells exhibiting a variety of, CK15/CK19 cellular phenotypes (CK15+/CK19+; CK15+/CK19−; CK15−/CK19+; CK15−/CK19−), often associated with simple columnar cells. Single layers of epithelial cells exhibiting all four CK15/CK19 phenotypes were observed contiguous to areas of atypical ductal hyperplasia that contained p53 positive cells that lost CK15 expression (CK15−/CK19+) and had a very similar phenotype to those of the neighboring ductal carcinoma *in situ* (DCIS) and invasive cells. The undifferentiated CK15+/CK19+ cells, which had the phenotype CK15+/CK19+/CK14+/CK8+ and −/ER −/PgR−/AR−/CD44+ (weak)/CK17−/p63−/vimentin+/Ki67−/Bcl-2+ (weak)/GATA-3−/p53−, most likely correspond to lineage-restricted luminal progenitor cells able to generate the other more differentiated CK15/CK19 cellular phenotypes, thus giving rise to the daunting intratumour heterogeneity displayed by carcinoma T71. Cells with a very similar phenotype to the CK15+/CK19+ progenitor cells were observed in a juvenile fibroadenoma as

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Abbreviations: IHC, immunohistochemistry; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis.
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well as in the large collecting ducts of the breast. The latter, however, expressed in addition CK14 and had a phenotype (CK15+/CK19+/CK14+/CK8+ (weak)/ER−/PgR−/AR−/CD44+ (weak)/CK17−/p63−/vimentin−/Ki67−/Bcl-2+/GATA-3−/p53−) that resembled that of the putative normal adult breast stem cells as inferred from published data. Further molecular characterization of these progenitor cells as well as unraveling of the signaling pathways that regulate their growth and differentiation may prove invaluable for developing novel therapeutic strategies that target cancer at an early stage.

1. Introduction

Breast cancer is the leading cause of cancer deaths in women today and is the most common cancer among women in the Western world. According to the World Health Organization at the turn of the century more than 1.2 million people were diagnosed with breast cancer worldwide (Parkin et al., 2005; Kamangar et al., 2006). In 2006 in Europe alone there were diagnosed an estimated 429,900 cases of breast cancer (13.5% of all cancer cases) (Ferlay et al., 2007). Currently, parameters such as axillary lymph node (ALN) status, tumour size, histological malignancy grade, and age, in combination with prognostic/predictive traits such as oestrogen and progesterone receptor status, and human epidermal growth factor receptor (HER-2/neu) status are used for selecting an appropriate therapeutic regimen (Goldhirsch et al., 2003; Hussain et al., 2007). As a result, adjuvant systemic therapy, chemotherapy and/or endocrine treatment (e.g. anti-estrogens) is offered to breast cancer patients who, due to the specific characteristics of their disease, are at risk of relapse and of eventually dying from the disease. This high-risk group constitutes approximately 70% of all new breast cancer patients, but it is a prognostically heterogeneous group with individual relapse risks varying widely, from as low as 10% to as high as 70%. Thus, although adjuvant systemic therapy has led to a considerable improvement in the prognosis of breast cancer patients, it also carries with it the significant adverse effect of overtreatment. Without a doubt, there is a need for new risk-stratification parameters that can guide medical decision making.

Towards this aim, whole-genome DNA micro array transcriptional profiling has been used to derive a molecular classification of breast cancer into clinically relevant subtypes, and sometimes to predict disease outcome and response to specific therapies (Perou et al., 2000; Sorlie et al., 2001; van de Vijver et al., 2002; van 't Veer et al., 2002; Goldhirsch et al., 2003; Sorlie, 2004; Sorlie, 2007; Sotiriou and Piccart, 2007). As a result of these studies, five different subtypes representing biologically distinct disease entities have been identified based on cell type origin and differentiation as well as HER-2 receptor status: these include basal-like, luminal A, luminal B, normal breast tissue-like, and ERBB2 positive (Sorlie, 2004). Besides reiterating that breast cancer is a complex and heterogeneous disease, these studies have highlighted the potential of the whole genome-profiling technologies to identify predictors of clinical outcome that in due course may be translated into a clinical assay. One such study, for example, identified a 70-gene expression predictor able to predict Distant Metastasis Free Survival (DMFS) (van 't Veer et al., 2002; Glas et al., 2006). A subsequent study validated the prognostic power of this molecular predictor and confirmed that this gene signature clearly separates a group with an excellent prognosis at 10 years from a group with a high risk of recurrence (Buyse et al., 2006). This molecular predictor is currently being validated by the Microarray In Node negative Disease may Avoid ChemoTherapy (MINDACT) trial; a multicentre, prospective, phase III randomised study comparing the 70-gene expression signature with a common clinical-pathological prognostic tool in selecting patients for adjuvant chemotherapy in node-negative breast cancer (Bogaerts et al., 2006). This broad patient stratification underlines the power of gene expression data in predicting general disease outcome, but at the same time emphasizes the need for classification of patients into more precise subgroups that are much more homogeneous with respect to disease outcome (Nevins et al., 2003).

One of the main problems associated with current whole-genome assays used for gene expression profiling of complex tissue samples, be it serial analysis of gene expression (SAGE), genomic microarrays, high-throughput RT-PCR, or proteomics, is that the expression patterns obtained by these technologies lack spatial resolution and as a result, are a composite derived from mixed cell populations due to tissue heterogeneity. Gene expression profiles from mixed cell populations reflect the phenotypes of major cell populations, being oblivious to rare cellular phenotypes. This limitation is particularly troublesome in the case of cancer given the increasing amount of data suggesting that most tumours may be derived from of a single cancer-initiating cell with stem cell properties, a cancer stem cell (CSC) (Al-Hajj et al., 2003; Dontu et al., 2005; Weissman, 2005a,b; Lynch et al., 2006; Polyak and Hahn, 2006; Clarke and Fuller, 2006; Lobo et al., in press; Sagar et al., 2007; Dalerba et al., 2007; Blanpain et al., 2007). CSCs are not necessarily the malignant counterparts of normal stem cells, but may arise from progenitor/amplified cells that can accumulate mutations and epigenetic changes in genes that regulate normal cell growth and differentiation and that play – together with the host microenvironment – a crucial role in tumour initiation and progression (Tysnes and Bjerkvig, 2007). Cancer initiating cells have the capacity of self-renewal giving rise to a copy of the stem cell as well as to a progenitor/amplifying cell that most likely generate the phenotypically heterogeneous cancer cells that form the bulk of the lesion (Polyak and Hahn, 2006; Blanpain et al.,

2007; Dalerba et al., 2007; Tysnes and Bjerkvig, 2007). CSCs have so far been described in acute myeloid leukaemia, and breast, brain, bone, lung, melanoma, prostate, and gastrointestinal cancer, although their expression features are at present largely unknown (Galli et al., 2004; Collins et al., 2005; Ponti et al., 2005; Weissman, 2005a,b; Polyak and Hahn, 2006; Clarke and Fuller, 2006; Lynch et al., 2006; Patrawala et al., 2006; Wicha et al., 2006; Dalerba et al., 2007; Zucchi et al., 2007). Molecular characterization of the phenotype(s) of breast stem cells and cancer initiating cells as well as the elucidation of the signalling pathways that regulate their growth and survival may be essential for comprehensive tumour classification and for developing novel therapeutic strategies, as current therapies are beset by relapses due to resistance (Dean et al., 2005; Eckfeldt et al., 2005). Moreover, the analysis of CSCs may lead to the identification of new predictive and prognostic markers.

Presently, only a few cell surface markers (e.g. CD44) are available to purify stem cells from dissociated mammary epithelium or tumours (Al-Hajj et al., 2003; Dontu et al., 2003), although intracellular markers are beginning to be identified that can delineate the phenotype of these cells. For example, Asselin-Labat et al. (2007) recently examined the expression profiles of normal mammary stems cells and their derivative colony forming progeny and reported that stem cells did not express estrogen (ER) or progesterone (PgR) receptors, ErbB2/Her2, or cytokeratin 18 (CK18, luminal marker), but expressed myoepithelial markers such as CK14 and p63 as well as epidermal growth factor receptor (EGFR). In addition, studies by Villadsen et al. (2007) in adult human breast tissue have revealed the existence of a stem cell hierarchy that expresses CK's 14 and 19, the specific embryonic antigen (SSA), as well as CK15, a cytoskeletal protein identified as a phenotypic marker of hair follicle bulge stem cells (Jih et al., 1999; Ohyama et al., 2006), skin keratinocyte stem cells (Webb et al., 2004), and limbal epithelial stem cells (Figueira et al., 2007).

In our laboratories we are interested in identifying precursor lesions of breast tumors as well as establishing approaches that will enable a more comprehensive classification of lesions for patient stratification. We routinely use proteomic analysis of a small-sized sample set of well-matched tissue biopsies for biomarker discovery, followed by immunohistochemistry (IHC) analysis on a larger sample set for confirmation of results (Celis et al., 2006a,b, 2007). Recently we presented evidence – based on the analysis of benign hyperproliferative lesions of the breast such as sclerosing adenosis with apocrine metaplasia – for the existence of putative luminal progenitor/amplified cells that expressed the stem cell marker CK15 in combination with CK19 (Celis et al., 2007). CK15+ cells were also observed to co-express CK14 in the myoepithelial lineage suggesting that this cytokeratin may be a neutral component whose cellular expression is permissive in progenitor cells that express multiple lineage-specific keratins. To determine the relationship of the CK15+/CK19+ cells with malignant disease, we performed an extensive gel-based proteomic and immunohistochemistry (IHC) analysis of high-risk breast carcinomas and non-malignant and pre-malignant lesions that we present here. Besides identifying a subset of breast carcinomas characterized by tumour-cell expression of CK15, our study revealed the existence of various phenotypes for putative progenitor cells that most likely generate the daunting cellular heterogeneity observed in some lesions.

2. Results

2.1. *CK15 is expressed by a subset of breast carcinomas*

Recently, we presented evidence – based on the analysis of benign hyperproliferative lesions of the breast such as sclerosing adenosis with apocrine metaplasia – for the presence of cells that express the epithelial stem cell marker CK15 in combination with CK19 (Celis et al., 2007), a cytokeratin that is widely expressed by breast carcinomas (Bartek et al., 1985; Ciocca et al., 2006). Given the fact that no association between the double positive CK15/CK19 cells and malignant disease was known and considering the significance of the relationship between stem/progenitor cells and cancer we decided to determine if breast carcinomas could present with this particular phenotype. Towards this end, a total of 120 carcinomas collected from mastectomies of high-risk breast cancer patients enrolled as part of a long-term translational programme at DCTB (Table 1) (Celis et al., 2004, 2005a,b, 2006a,b,c) were analyzed by IHC for the concomitant expression of CK15 and CK19 using monoclonal antibodies that specifically recognize these cytokeratins as determined by 2D gel Western blotting (Celis et al., 2007). The results, which are based on the analysis of several independently stained sections of formalin-fixed paraffin-embedded samples, showed that only the carcinoma from patient 65 contained a substantial number of tumour cells that stained positively with both antibodies (arrows in Fig. 1A,B). In some areas of this sample, however, we also observed invasive cells that expressed only CK15 (Fig. 1C,D; red arrows) or CK19 (Fig. 1C,D; blue arrows), suggesting that under certain conditions the expression of these cytokeratins may be mutually exclusive (Celis et al., 2007). Analysis of normal looking ducts present in areas very close to the tumour also revealed cells with the phenotypes CK15+/CK19+, CK15+/CK19−, CK15−/CK19− (not shown, but see Fig. 9). In addition to the carcinoma from patient 65, the IHC analysis identified four carcinomas with tumour cells that expressed CK15, but not CK19; these included tumours from patients 23 (Fig. 1E,F), 42 (Fig. 1I,J), 66 (Fig. 1K,L), and 88 (results not shown). Tumour cells in the remaining 115 carcinomas analyzed were negative for CK15, but positive for CK19: this is exemplified in Fig. 1M,N with the carcinoma from patient 25 stained with the CK15 (Fig. 1M) and CK19 (Fig. 1N) antibodies, respectively.

Tumours 65 (grade 1, ER+/PgR+, Her-2 neu −) and 66 (grade 2, ER+/PgR+, Her-2 neu −) were classified as low grade lobular carcinomas, and of the two cases only patient 65 presented with ALN metastases (Table 1). Both lobular tumours were E-cadherin negative, a frequently observed phenotype in tumours of lobular subtype (Berx et al., 1996), as determined by 2D gel analysis, immunoblotting, and IHC (results not shown). Tumours 23 (grade 3, ER−/PgR, Her-2 neu +), 42 (grade 3, ER−/PgR, Her-2 neu 2−), and 88 (grade 2, ER+/PgR+, Her-2 neu 2−), on the other hand, were diagnosed as high histological grade carcinomas of the ductal type and all three patients

Table 1 – High-risk breast cancer patients

Patient number	Age (years)	Tumor type[a]	Tumor size[b]	Grade[c]	Her2-neu IHC[d]	Her2-neu FISH[d]	ALN[e]	Receptor test[f]	
1	66	D	17	2	0		N+ 2/18	ER+	PgR+
2	53	D	30	2	3+		N− 0/16	ER+	PgR−
3	84	D	17	3	2+	2.3	N+ 1/17	ER+	PgR−
4	44	D	17	1	0		N+ 4/16	ER+	PgR+
5	73	D	21	1	0		N+ 2/13	ER+	PgR+
6	72	D	21	3	3+		N− 0/12	ER+	PgR−
7	54	D	30	1	1+		N+ 9/20	ER+	PgR+
8	93	D	70	2	1+		N+ 5/10	ER+	PgR−
9	80	D	28	3	3+		N− 0/0	ER−	PgR−
10	45	L	30	1	0		N− 0/14	ER+	PgR+
11	57	L	30	1	1+		N− 0/14	ER+	PgR+
12	47	D	35	3	0		N+ 23/23	ER+	PgR+
13	54	D	19	1	0		N+ 4/28	ER+	PgR+
14	39	D	33	3	0		N+ 5/15	ER−	PgR−
15	60	D	30	3	3+		N+ 11/15	ER+	PgR−
16	50	D	41	3	0		N+ 3/13	ER−	PgR−
17	76	D	85	2	3+		N+ 4/14	ER+	PgR+
18	85	D	30	2	2+	1.5	N+ 6/10	ER+	PgR+
19	67	D	10	1	0		N+ 1/11	ER+	PgR+
20	91	D	23	2	1+		N+ 4/15	ER+	PgR+
21	73	D	30	2	2+	1.7	N+ 5/10	ER+	PgR+
22	54	D	50	3	1+		N+ 1/22	ER−	PgR−
23	63	D	40	3	3+		N+ 7/16	ER−	PgR−
24	44	D	27	1	2+	1.7	N+ 9/11	ER+	PgR+
25	62	D	20	2	2+	1.6	N+ 2/7	ER+	PgR+
26	71	Muc	28	2	1+		N− 0/16	ER+	PgR+
27	47	D	20	2	0		N− 0/15	ER+	PgR+
28	53	D	24	3	3+		N+ 3/12	ER+	PgR+
29	45	D	13	2	0		nd 0/0	ER+	PgR+
30	69	D	26	2	3+		N+ 1/6	ER+	PgR+
31	89	D	22	1	1+		N+ 6/15	ER+	PgR+
33	68	D	21	2	3+		N+ 1/11	ER+	PgR+
34	69	D	23	2	1+		N+ 3/12	ER+	PgR+
35	75	L	40	1	1+		N+ 1/16	ER+	PgR+
36	48	D	50	2	3+		N+ 8/18	ER+	PgR+
37	80	D	16	1	0		N− 0/1	ER+	PgR−
38	62	D	25	2	2+	3.1	N+ 2/13	ER+	PgR−
39	73	D	25	1	2+	1.0	N− 0/13	ER+	PgR−
40	52	D	30	2	2+	1.9	N− 0/14	ER+	PgR+
41	68	D	35	3	3+		N+ 1/18	ER−	PgR−
42	54	D	21	3	2+	1.6	N+ 5/10	ER−	PgR−
43	55	L	30	1	1+		N− 0/13	ER+	PgR−
44	42	D	70	2	1+		N+ 7/11	ER+	PgR+
45	65	D	28	1	2+	1.8	N+ 4/14	ER+	PgR+
46	65	D	30	3	2+	1.2	N+ 3/19	ER−	PgR−
47	47	D	20	2	2+	2.21	N+ 4/12	ER+	PgR+
48	61	D	21	1	2+	1.59	N− 0/11	ER+	PgR+
49	78	D	35	2	2+	1.65	N+ 6/7	ER+	PgR−
50	46	D	26	2	3+		N+ 2/10	ER+	PgR+
51	27	D	40	3	2+	1.49	N+ 4/26	ER+	PgR+
52	80	L	40	2	3+		N− 0/11	ER−	PgR−
53	51	D	21	1	1+		N+ 2/11	ER+	PgR+
54	88	D	40	1	1+		N+ 1/7	ER+	PgR−
55	46	D	50	2	0		N+ 4/22	ER+	PgR+
56	44	L	20	1	1+		N+ 1/14	ER+	PgR−
57	66	D	30	3	3+		N+ 27/31	ER−	PgR−
58	38	D	20	3	3+		N+ 9/20	ER−	PgR−
59	38	D	25	1	1+		N− 0/15	ER+	PgR+
60	72	D	30	2	2+	1.2	N+ 3/14	ER+	PgR−
61	99	D	40	2	2+	1.5	N+ 7/11	ER+	PgR+
62	85	L	25	2	2+	1.31	N+ 1/13	ER+	PgR+
63	92	D	16	2	2+	1.46	N− 0/13	ER+	PgR+
64	52	Tu/Crib	23	1	1+		N+ 23/25	ER+	PgR+
65	81	L	50	1	1+		N+ 4/15	ER+	PgR+
66	60	L	70	2	1+		N− 0/10	ER+	PgR+
67	78	L	22	2	0		N− 0/7	ER+	PgR+

Table 1 (*continued*)

Patient number	Age (years)	Tumor type[a]	Tumor size[b]	Grade[c]	Her2-neu IHC[d]	Her2-neu FISH[d]	ALN[e]	Receptor test[f]	
68	51	L	35	2	1+		N+ 2/12	ER+	PgR+
69	86	D	25	2	0		N+ 3/15	ER+	PgR+
70	83	D	33	3	3+		N+ 3/11	ER−	PgR−
71	40	D	50	3	3+		N− 0/21	ER−	PgR−
72	52	D	25	3	2+	2.75	N+ 1/15	ER+	PgR+
73	75	L	50	1	1+		N+ 14/15	ER+	PgR−
74	49	D	21	3	1+		N+ 3/22	ER−	PgR−
75	62	D	20	3	3+		N+ 9/14	ER−	PgR−
76	76	D	30	2	2+	1.21	N− 0/20	ER+	PgR−
77	62	L	30	2	2+	1.39	N+ 11/17	ER+	PgR−
78	31	D	32	3	0		N+ 14/17	ER−	PgR−
79	56	Apocrine	35	1	1+		N− 0/17	ER−	PgR−
80	41	D	40	3	3+		N+ 1/15	ER+	PgR+
81	39	L	50	2	1+		N+ 8/15	ER+	PgR+
82	72	D	25	2	2+	2.18	N+ 1/15	ER+	PgR+
83	57	D	45	2	2+	1.19	N+ 10/16	ER+	PgR+
84	38	D	18	2	0		N+ 3/11	ER+	PgR+
85	80	D	30	2	2+	1.69	N+ 3/16	ER+	PgR−
86	77	D	110	2	1+		N+ 20/20	ER+	PgR+
87	66	D	25	3	3+		N− 0/10	ER−	PgR−
88	83	D	35	2	2+	1.48	N+ 8/15	ER+	PgR+
89	43	D	16	3	0		N+ 1/13	ER+	PgR−
90	49	D	40	3	3+		N+ 15/16	ER−	PgR−
91	59	D	30	2	3+		N+ 13/16	ER+	PgR−
92	49	D	27	3	2+		N− 0/10	ER+	PgR−
93	60	D	35	2	1+		N+ 11/14	ER+	PgR+
94	57	D	21	3	3+		N+ 3/13	ER−	PgR−
95	44	L	25	1	1+		N+ 4/16	ER+	PgR+
96	60	D	12	1	2+	1.41	N− 0/2	ER+	PgR+
97	45	D	25	3	2+	1.6	N+ 4/12	ER+	PgR+
98	51	D	45	2	1+		N+ 2/14	ER+	PgR+
99	43	D	40	2	1+		N+ 8/14	ER+	PgR+
100	85	D	20	2	2+	2.1	N− 0/2	ER+	PgR+
101	45	D	22	2	2+	1.44	N+ 3/11	ER+	PgR+
102	73	D	60	2	0		N+ 1/11	ER+	PgR−
103	71	D	25	1	0		N+ 8/16	ER+	PgR+
104	67	D	55	1	1+		N+ 12/12	ER+	PgR+
105	83	D	60	2	0		N+ 13/18	ER+	PgR+
106	86	D	22	2	0		N− 0/7	ER+	PgR+
107	45	D	15	3	3+		N+ 2/20	ER−	PgR−
108	87	D	25	2	3+		N+ 3/13	ER+	PgR−
109	79	D	20	1	0		N+ 4/10	ER+	PgR+
110	82	D	40	2	2+	0.14	N+ 18/18	ER+	PgR−
111	51	D	23	2	3+		N+ 3/9	ER−	PgR−
112	46	D	15	2	0		N− 0/15	ER+	PgR+
113	83	D	15	3	3+		N+ 20/20	ER−	PgR−
115	79	L	22	2	1+		N+ 4/10	ER+	PgR+
116	84	D	60	3	2+		N+ 19/22	ER+	PgR−
117	74	D	30	3	1+		N+ 8/15	ER+	PgR+
118	83	L	33	2	1+		N+ 10/12	ER+	PgR+
119	79	D	35	3	1+		N+ 10/10	ER+	PgR+
120	62	D	17	3	0		N+ 3/9	ER−	PgR−
121	33	D	25	2	2+		N− 0/5	ER+	PgR+
122	73	D	18	1	1+		N+5/10	ER+	PgR+

The criteria for high-risk cancer applied by the Danish Cooperative Breast Cancer Group (DBCG) are age below 35 years old, and/or tumor diameter of more than 20 mm, and/or histological malignancy 2 or 3, and/or, negative estrogen and progesterone receptor status, and/or positive axillary status.

a D, Ductal carcinoma. L, Lobular carcinoma. Muc, Mucinous. Tu/Crb, Tubular/Cribriform.

b The tumor size is given in mm.

c The histological malignancy grade was determined according to Elston and Ellis (1991).

d HER2 positive: IHC staining 3+, or 2+ if amplified, i.e. FISH HER2/CEN-17 ratio >2.

e ALN, axillary lymph node. N+, metastasis in lymph nodes. N−, no metastasis detected in lymph nodes (see also Section 4), nd, not determined.

f ER, estrogen receptor. PgR, progesterone receptor. ER and PgR status was determined by IHC analysis according to DBCG guidelines. Tumors were regarded as negative when both receptors were expressed in less than 10% of tumor cell nuclei.

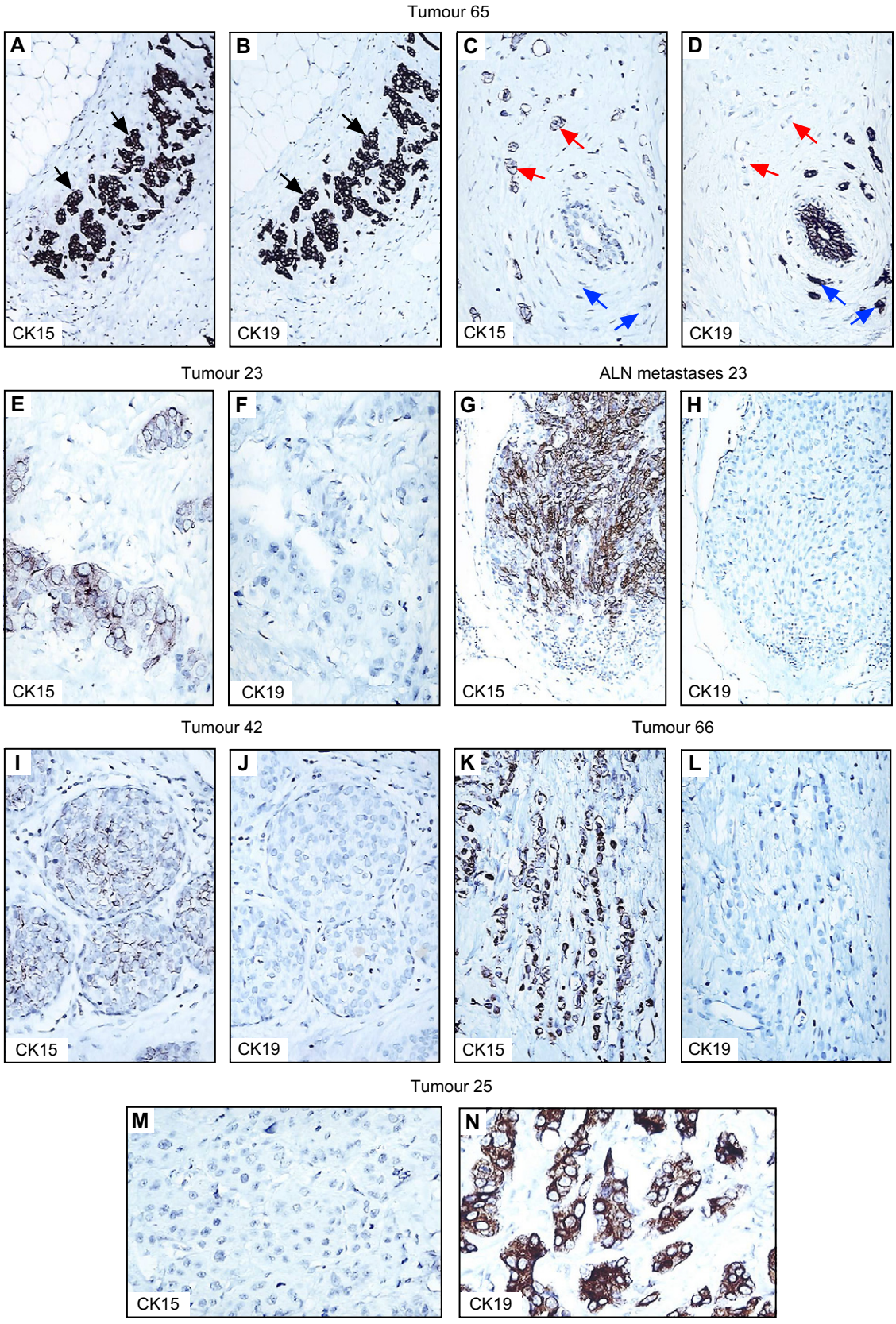


Figure 1 – IHC pictures of serial paraffin-embedded sections of tumours and ALN metastases stained with antibodies against CK15 (monoclonal) and CK19. Black arrows in (A) and (B) indicate cells that express both CK15 and CK19. Red arrows in (C) and (D) indicate cells that express only CK15, while blue arrows indicate cells that express only CK19.

exhibited ALN metastases (Table 1). IHC staining of the ALN metastases of patients 23 and 88 showed that the cells were CK15 positive (Fig. 1G; ALN M23) and CK19 negative (Fig. 1H; ALN M23), just like in the primary tumour. There was not enough tissue available, however, to carry out a similar analysis of ALN metastases of patients 42 and 65.

From the IHC data obtained for the 120 carcinomas analyzed it is clear that there are several permissible CK15/CK19 phenotypes (CK15+/CK19+; CK15+/CK19−; CK15−/CK19+) that can be expressed by the tumour cells, of which the CK15−/CK19+ one was by far the most common. The data also showed that all three phenotypes co-existed in the same tumour (T65), highlighting the intratumour heterogeneity within this lesion.

To confirm the IHC data, we analyzed fresh frozen tumour tissue biopsies from all 120 patients by means of high-resolution 2D-gel electrophoresis in combination with mass spectrometry for protein identification. As seen in the silver stained gels presented in Fig. 2, the carcinoma from patient 65 expressed both CK15 and CK19 (Fig. 2A; relatively lower levels of CK19), while those from patients 23 (Fig. 2B) and its ANL metastases (Fig. 2C), 42 (Fig. 2D), 66 (Fig. 2E), and 88 (results not shown) expressed only CK15 in line with the IHC data. For comparison, Fig. 2F shows a representative 2D gel of the carcinoma from patient 25 that is CK19 positive, but CK15 negative. Expression of CK19 and lack of expression of CK15 by the other 115 carcinomas analyzed (Table 1) was confirmed by similar gel-based proteome analysis (results not shown), except in the case of tumour 57, a high grade, highly metastatic ductal carcinoma (grade 3, ER−/PgR−, Her-2 neu +; Table 1) that showed relatively low level expression of CK15 and high levels of CK19 (Fig. 3A). In contrast, abundant levels of both cytokeratins were observed in the matched ALN metastases (Fig. 3B). IHC staining of the metastases with the CK15 antibody confirmed the presence of CK15 in all cells, but very weak or no staining was observed in the epithelial cells present in the tumour (results not shown). To resolve this apparent discrepancy we repeated our analysis using a polyclonal anti-CK15 antibody raised against an internal region of CK15 (Aviva Systems Biology). This antibody is also highly specific for CK15 (Fig. 3C) and generally yields stronger staining than the monoclonal antibody, which recognizes an epitope from the C-terminal region of CK15 (clone LHK15). As shown in Fig. 3D, the polyclonal antibody reacted weakly with a few cells in the tumour, while all cells in the ALN metastases reacted strongly (Fig. 3E). These results underlie a general problem associated with sampling and reactivity of the antibodies, as it is likely that the CK15 cells were present only in a fraction of the tumour and distributed unequally within it. One important corollary of this analysis, however, is that the results implied that the ALN metastases in this patient arose from the minor fraction of CK15 positive tumour cells rather than from the CK15 negative ones that formed the bulk of the lesion.

As a whole, the experiments showed a very good correlation between the IHC and 2D PAGE data, and indicated that the sectioning procedure we used for sample preparation (Section 4), which allowed us to keep a record of the histology of the samples being analyzed, may in some way alleviate the problems imposed by sampling.

2.2. *Identification of CK15 positive non-malignant precursor cells in carcinoma T71: intratumour heterogeneity and relation with pre-malignant lesions and invasive disease*

The results presented above together with previous studies from our laboratory (Celis et al., 2007) suggested that the expression of CK's 15 and 19 may be mutually exclusive under some conditions. In addition, they raised questions about the nature of the progenitor cells involved in generating the CK15/CK19 intratumour heterogeneity observed in carcinoma 65 (Fig. 1A–D). To clarify this issue we searched among our sample set for tumours in which invasive cancer co-existed with non-malignant epithelial cells, atypical ductal hyperplasia (ADH)[2] and ductal carcinoma *in situ* (DCIS), as we surmised that some of these cells/lesions may have lost the capacity to express CK15 as a result of tumour initiation and/or progression, and thus may be instrumental in shedding some light as to the phenotype of the progenitor/precursor cells involved. Three carcinomas that satisfied this criterion were found, but only tumour 71 contained CK15 positive cells in non-malignant cells and in areas with hyperplasia of the usual type (HUT).[3] Single cell layers of CK15 positive cells were often seen adjacent to ADHs with p53 positive cells, suggesting that these cells may play a role in the development of pre-malignant lesions.

According to the Pathology report, tumour 71 consisted of a 50 mm large central tumour area that contained multiple and closely located in ductal *in situ* elements of the Comedo type i.e. grade 3 with centred necrosis (Silverstein et al., 1995), as well as areas with invasion intermingled with numerous intracystic papillomas with multiple branching papillae. A morphological spectrum of benign ducts with simple columnar cell change were observed throughout the sample (Simpson et al., 2005; Pinder and Reis-filho, 2006; Dabbs et al., 2006). The tumour cells, which were of malignancy grade 3, were located in small islands in defined areas of the tumour mass. The breast tissue around the tumour also contained hyperplasia, fibroadenoma, adenosis, and papillomas with apocrine metaplasia. The tumour cells were p53 positive, ER and PgR negative, Her-2 neu 3+, and AR positive (Tables 1 and 2). Fig. 4A,B show serial sections of an area of tumour 71 exhibiting HUT, ADH (Page, 1991), invasive disease, and papillary lesions with apocrine metaplasia stained with the CK15 (Fig. 4A) and CK19 (Fig. 4B) antibodies, respectively. Four CK15/CK19 cellular phenotypes were observed in the non-malignant epithelial areas of the sample: (i) CK15+/CK19+; (ii) CK15+/CK19−; (iii) CK15−/CK19+; and (iv) CK15−/CK19− (Fig. 4A,B; representative areas are indicated for reference). The invasive tumour area was CK15−/CK19+ as indicated in Fig. 4A,B. IHC analysis of several sections of the tumour revealed considerable variation in the structures present in

[2] "The proliferating epithelial cells display a cytological atypia similar to the cytological features of one of the non-necrotic variants of CIS" (Tavassoli and Norris, 1994).

[3] "The World Health Organization (WHO) defined HUT as a benign ductal proliferative lesion characterized by secondary lumens and streaming of the proliferating cells. The lesions show great variability of cells and nuclei yet not overtly malignant nuclear features" (The World Health Organization, 2003).

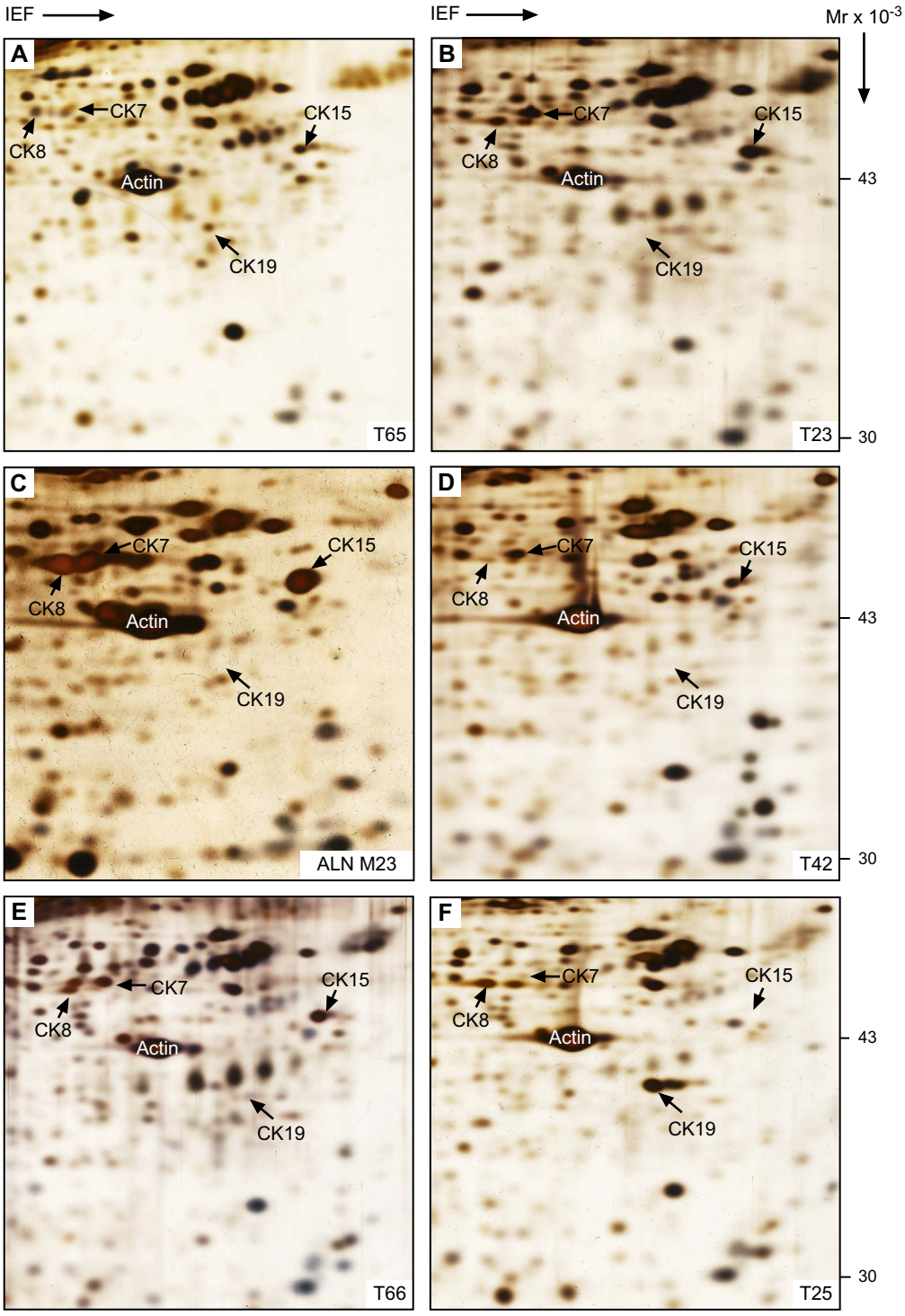


Figure 2 – IEF 2D PAGE separation of whole protein extracts from tumour and ALN metastases. Gels were stained with silver nitrate as previously described (Gromova and Celis, 2006). The identity of the various cytokeratins (CK's 7, 8, 15 and 19) was determined by mass spectrometry as described in Section 4. Mr, apparent molecular weight.

the sections, in particular the number of CIS and papillary lesions. A representative 2D gel pattern of a sample originating from an area of the tumour located very close to the one indicated in Fig. 4, showing similar morphological features, is presented in Fig. 5A; as expected both CK15 and CK19 were present in the gels, a fact that supported the IHC analysis. A similar 2D gel pattern of a sample derived from a region enriched for CIS of the Comedo type is shown in Fig. 5B for reference. These lesions did not express CK15, but were CK19 positive.

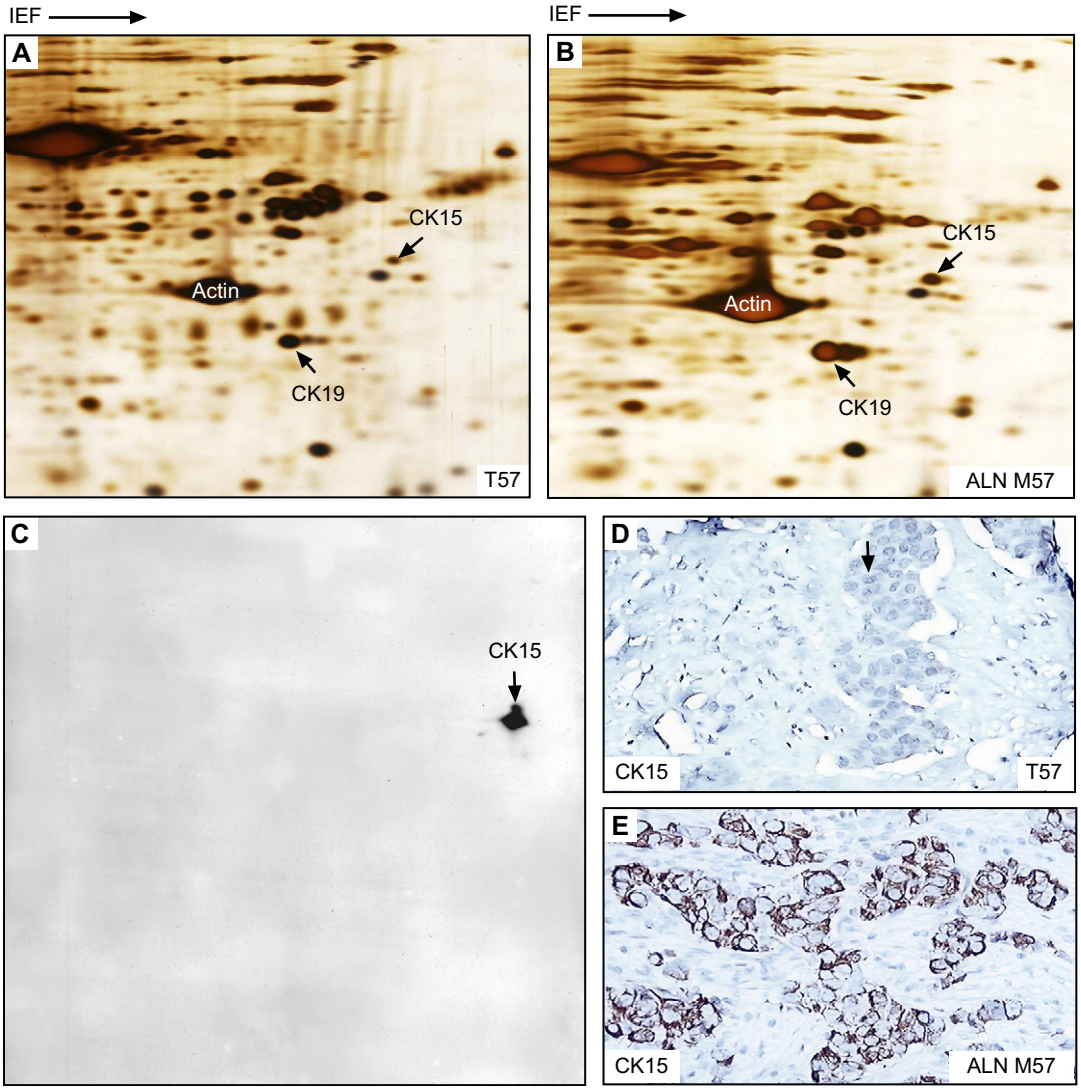


Figure 3 – IEF 2D PAGE and IHC analysis of T57 and the corresponding ALN metastases. (A) and (B), 2D PAGE separation of whole protein extracts from T57 and ALN metastases 57. (C) 2D gel Western blot of whole proteins from non-cultured keratinocytes reacted with the Aviva anti-CK15 polyclonal antibody. (D) and (E) IHC of paraffin-embedded sections from T57 and ALN M57 reacted with the AVIVA CK15 antibody. The black arrow in (D) indicates a cell that stained weakly with the antibody.

In an effort to gain a better understanding of the relationship between the various CK15/CK19 phenotypes present in non-malignant cells, premalignant lesions, and invasive disease we describe below a detailed IHC analysis of tumour 71 using a battery of antibodies against luminal and myoepithelial cytokeratins (CK's 7, 8, 14, 15, 17, 18 and 19) (Moll et al., 1982; Hesse et al., 2004; Schweizer et al., 2006), receptors (ER, PgR and AR) (Klijn et al., 1993; Birrell et al., 1998), and markers for stem cells (CD44) (Al-Hajj et al., 2003; Dontu et al., 2003), myoepithelial cells (p63, vimentin) (Lerwill, 2004; Polyak and Hu, 2005; Adriance et al., 2005; Moriya et al., 2006) cell proliferation (Ki67) (Vartanian and Weidner, 1994; van Diest et al., 2004), and apoptosis (Bcl-2) (Tsujimoto et al., 1984; Adams and Cory, 2007), as well as a factor that maintains luminal epithelial differentiation (GATA-3) (Kouros-Mehr et al., 2006; Asselin-Labat et al., 2007), and proteins overexpressed by some breast carcinomas (p53, psoriasin (S100A7), and MRP14 (S100A9)) (Clahsen et al., 1998; Arai et al., 2004; Emberley et al., 2004; Carlsson et al., 2005; Krop et al., 2005; Lacroix, 2006; Rohan et al., 2006; Herceg and Hainaut, 2007; Skliris et al., 2007). Moreover, we describe related phenotypes of double positive CK15/CK19 cells found in the large central collecting ducts of the nipple and in a juvenile fibroadenoma with epithelial hyperplasia (Table 3).

2.2.1. *CK15 positive non-malignant and malignant epithelial cells present in Tumour 71*

2.2.1.1. *Single cells luminal epithelia*

2.2.1.1.1. *CK15+/CK19+ cells.* A few areas in tumour 71 contained non-malignant luminal cells co-expressing CK's 15 and 19 (Fig. 4A,B); one such representative area stained with the

Table 2 – Phenotype of CK15 positive and negative cells in non-malignant cells and malignant lesions of patient 71

Cells/Lesions	Markers																		
	Cytokeratins					Receptors			Stem cell	Myoephitelial cell markers				Proliferation	Apoptosis	Differentiation	Tumor associated		
	CK15	CK19	CK7	CK8	CK18	ER	PgR	AR	CD44	CK14	CK17	p63	Vimentin	Ki67	Bcl-2	GATA-3	p53	psoriasin (S100A7)	MRP14 (S100A9)
1. Non-malignant cells																			
CK15+/CK19+ (Progenitor-like)	Pos	Pos	Pos	Neg & Pos[a]	Neg & Pos[b]	Neg[c]	Neg[c]	Neg[c]	Pos[d] (weak)	Neg	Neg	Neg	Pos[e]	Neg[f]	Pos (weak)	Neg[c]	Neg	Neg	Neg
CK15+/CK19−	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg[g]	Pos	Pos	Neg	Neg	Neg
CK15−/CK19+	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos[d] (weak)	Neg	Neg	Neg	Neg	Neg[f]	Pos	Pos	Neg	Neg	Neg
CK15−/CK19−	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos (weak)	Pos[d] (weak)	Neg	Neg	Neg	Neg	Neg[f]	Pos	Neg	Neg	Neg	Neg
2. Ductal hyperplasia of the usual type (HUT)																			
HUT 1[r]	Pos	Pos	Pos[h]	Neg & Pos[a]	Neg & Pos[b]	Neg	Neg	nd[i]	Pos	Neg	Neg	Neg	Pos	Pos[j]	Pos	Neg	Neg	Neg	Neg
HUT 2[s]	Pos	Neg	Pos[h]	Pos[g]	Pos	Pos	Pos	nd[i]	Pos	Neg	Neg	Neg	Pos	Pos[j]	Pos	Pos	Neg	Neg	Neg
3. Atypical ductal hyperplasia																			
ADH 1[t]	Neg	Pos	Pos[h]	Pos[k]	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Pos[l]	Neg	Neg	Pos	Some Pos[m]	Very Few Pos[n]
ADH 2[u]	Neg	Pos	Pos[h]	Pos[k]	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Pos	Pos[l]	Neg	Neg	Pos	Some Pos[m]	Very Few Pos[n]
4. Carcinoma *in situ*																			
CIS	Neg	Pos	Pos[h]	Pos[o]	Neg	Neg	Neg	Pos[p]	Pos	Neg	Neg	Neg	Pos[q]	Pos[l]	Neg	Neg	Pos	Pos	Very Few Pos[n]
5. Tumour																			
Invasive lesion	Neg	Pos	Pos[h]	Pos[o]	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Pos[l]	Neg	Neg	Pos	Pos	Pos

a A few positive cells intermingling with negative ones were observed (see also Fig. 6D; positive cells are indicated with arrows).
b Very few positives cells were observed, just as in the case of CK8.
c Only a few positive cells were detected (less than 10%; see Fig. 6E).
d Positive, but the intensity was weaker than that observed in CIS cells.
e Only very few vimentin negative cells were observed (arrows in Fig. 6G).
f Less than 0.5% of the cells stained with the antibody.
g There were a few areas, however, where as many as 20% of the cells were positive.
h Positive, but weaker than in CK15+/CK19+ cells (see Fig. 6C).
i nd, not determined.
j About 5% of the cells stained with the antibody.
k Positive, but attenuated as in the case of CIS and invasive cells.
l 30% or more of the cells were positive.
m Less than 10% of the cells (see Fig. 7L).
n Only a few scattered cells were observed.
o Attenuated: positive, but less intensive than the staining observed in CK15−/CK19+ cells.
p There are a few CIS that are AR negative.
q About 50% of the CIS are vimentin positive.
r Adjacent to CK15+/CK19+ non-malignant cells.
s Adjacent to CK15+/CK19− non-malignant cells.
t Cells with all four CK15+/CK19+; CK15+/CK19−; CK15−/CK19+; CK15−/CK19− were at times observed contiguous to ADHs. We also observed a few ADHs that were p53 positive, vimentin positive and CK15 negative, but those were a minority.
u These lesions were less abundant than the ADH 1.

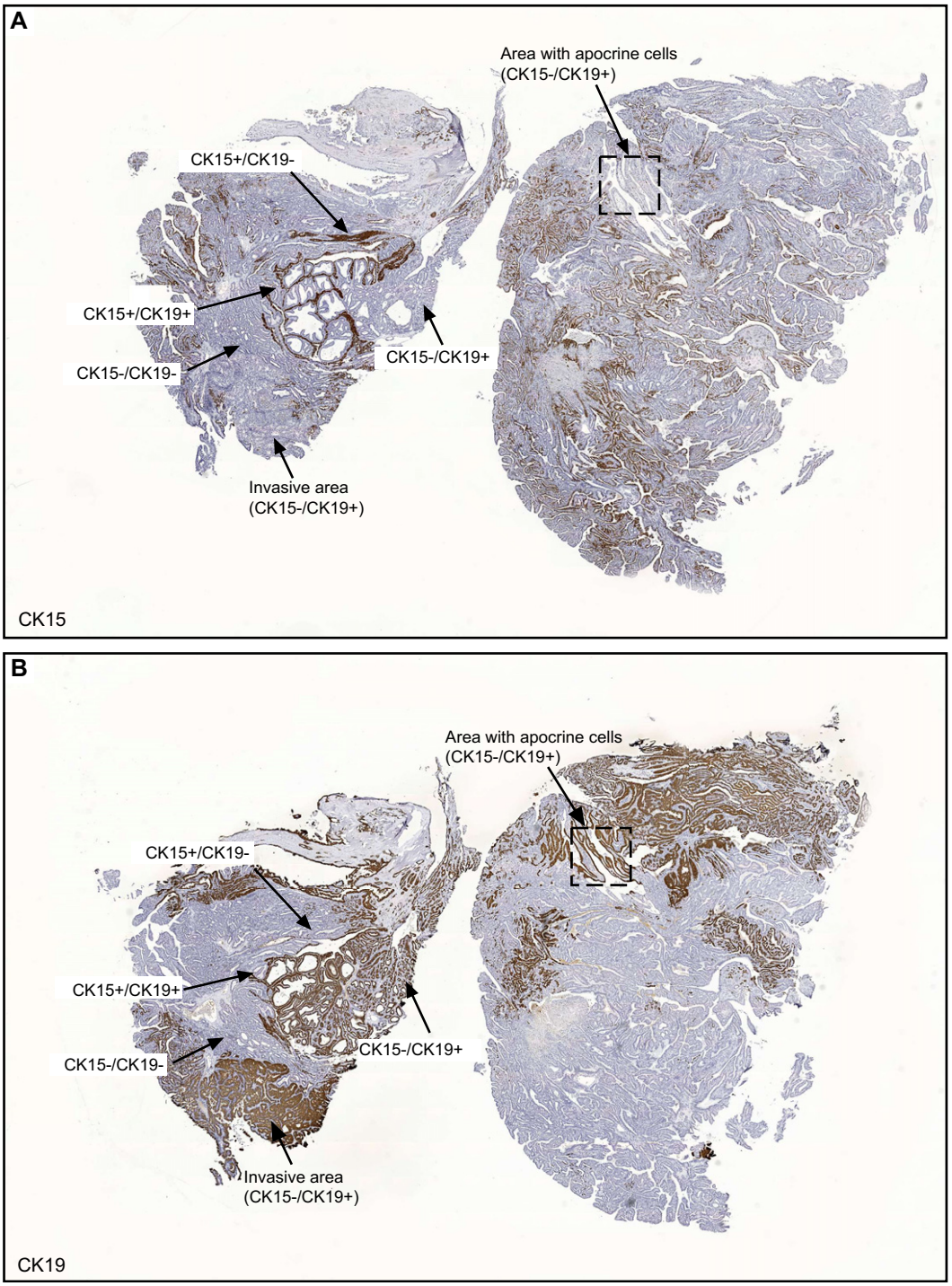


Figure 4 – IHC staining of serial paraffin-embedded sections of tumour 71 stained with antibodies against (A) CK15 (monoclonal) and (B) CK19. Areas with cells exhibiting different CK15/CK19 phenotypes are indicated for reference. A region containing apocrine cells as judged by the distinct morphological features of these lesions is indicated within a box. The heterogeneity of the tumour in terms of CK15/CK19 phenotypes is clearly illustrated in these sections.

CK15 antibody is shown in Fig. 6A in Panel A. Based on the IHC analysis of consecutive serial sections from several formalin-fixed paraffin-embedded preparations obtained from different areas of the tumour we were able to establish the phenotype for the double positive CK15/CK19 cells presented in Table 2 and illustrated in Fig. 6 with a few examples: CK7 positive (Fig. 6C), CK8 negative or positive (Fig. 6D; intermingling negative and positive cells), CK18 negative or positive as in the case of CK8, ER negative, PgR negative (Fig. 6E), AR negative, CD44 weakly positive, CK14 negative (Fig. 6F), CK17 negative, p63 negative, vimentin positive (Fig. 6G; red arrows indicate a few vimentin negative cells), Ki67 negative, Bcl-2 weakly positive,

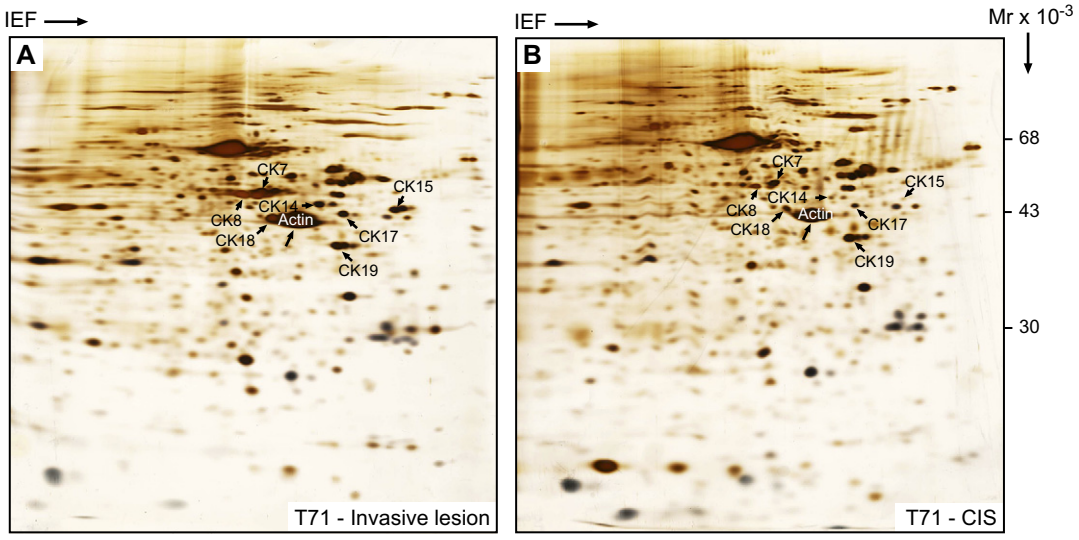


Figure 5 – IEF 2D PAGE separation of whole protein extracts from the invasive area of tumour 71 (A) and an area enriched in CIS of the Comedo type (B). The identity of the various cytokeratins indicated (CK's 7, 8, 14, 15, 17, 18 and 19) was assessed by mass spectrometry as described in Section 4. Mr, apparent molecular weight.

GATA-3 negative, p53 negative, psoriasin (S100-A7) negative, and MRP14 (S100A9) negative. In general, however, we observed slight deviations to the phenotype listed in Table 2, as in some cases we could observe differences in the expression of a given antigen by some cells within the same duct or lobule. This is illustrated in Panel B of Fig. 6 which shows serial sections of a TDLU in carcinoma 71 showing that CK15+/CK19+ cells co-exist with CK15−/CK19+ cells in these structures. While the few CK15 negative cells are CK19, CD44 and GATA-3 positive (red arrows), the undifferentiated CK15+ ones which are the most abundant, are CK19 and CD44 positive, but negative for GATA-3 (blue arrows).

As a whole the CK15+/CK19+ cells were undifferentiated as judged by the lack of expression of GATA-3, receptor negative, and the majority was quiescent as judged by the expression of Ki67, a well-known marker of cell proliferation. The cells also expressed Bcl-2, an anti-apoptotic protein whose loss has been associated with deregulated proliferation (Binder et al., 1995) and aggressiveness (Silvestrini et al., 1996) in breast cancer. Interestingly, some of the cells had an elongated morphology and projected towards the lumen suggesting that they have migratory capacity.

2.2.1.1.2. *CK15+/CK19− cells*. Differentiated CK15+/CK19− cells as judged by the expression of GATA-3, were strongly positive for CK's 8 and 18, expressed the ER, PgR and AR receptor, were CK14 and vimentin negative, and Bcl-2 positive, and negative for p53 (Table 2). In general, CK15+/CK19− cells were quite abundant and were quiescent, although there were a few regions in the preparations in which about 20% of the cells stained positively with the Ki67 antibody.

In some areas of the sections, we also observed single layers of CK15+/CK19− columnar cells (Fig. 7A, blue arrows), contiguous to cells undergoing apocrine metaplasia as defined by histological morphology as well as by staining with COX-2 (Fig. 7B, red arrows) and other antibodies, namely 15-prostaglandin dehydrogenase (15-PGDH), specific for these cells (not shown) (Celis et al., 2006a,b). Apocrine metaplasia is caused by reprogramming of the CK15+/CK19− cells leading to cells that are cytologically identical to those in apocrine glands in that they exhibit rather large vesicular nuclei with prominent nucleoli, as well as abundant eosinophilic cytoplasm that occasionally present apical snouts that are shed into the lumen of the ducts (Wellings and Alpers, 1987; Jones et al., 2001; Celis et al., 2006a,b; Tysnes and Bjerkvig, 2007).

2.2.1.1.3. *CK15−/CK19+ cells*. Differentiated CK15−/CK19+ cells were GATA-3 positive and were relatively abundant. They were positive for CK's 8 and 18, expressed the PgR and AR receptor, were CK14 and vimentin negative, Bcl-2 positive, and p53 negative (Table 2).

2.2.1.1.4. *CK15−/CK19− cells*. Cells exhibiting the CK15−/CK19− phenotype were less abundant than the other three phenotypes (Fig. 4). These cells were differentiated as judged by the expression of GATA-3, were positive for CK8 and CK18, expressed ER and PgR, stained weakly with the AR antibody and were negative for CK7, CK14, vimentin and p53, but positive for Bcl-2 (Table 2).

2.2.1.2. *Hyperplasia of the usual type (HUT), atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and invasive cells*

2.2.1.2.1. *HUTs*. Two types of HUTs (HUTs 1 and 2) were identified based on their IHC phenotypes as indicated in Table 2. HUT 1, which were found adjacent to CK15+/CK19+ cells, expressed CK's 15 and 19 (Panel A, Fig. 8A,B), were vimentin positive (Fig. 8C), CK8 negative or positive, ER/PgR negative, CD44 positive, Ki67 positive, Bcl-2 positive, GATA-3 negative, p53 negative (Fig. 8D) and psoriasin (S100A7) and MRP14 (S100A9) negative (Table 2). HUT 2; on

Table 3 – Phenotypes of CK15 positive and negative cells in large collecting ducts and benign lesions of the breast

Sample	Markers[a]													
	CK15	CK19	CK8	ER	PgR	AR	CD44	CK14	CK17	p63	Vimentin	Ki67	Bcl-2	GATA-3	p53
1. Non-malignant cells in large collecting ducts (Nipple)															
Phenotype 1 (collecting duct progenitor)	Pos	Pos	Pos[b] (weak)	Neg[c]	Neg[c]	Neg[c]	Pos (weak)	Pos	Neg	Neg	Neg[d]	Neg[c]	Pos	Neg	Neg
Phenotype 2	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg[c]	Pos	Pos	Neg
2. Benign conditions															
a) Juvenile fibroadenoma															
Phenotype 1	Pos	Pos	Pos (weak)	Neg[c]	Neg[c]	Neg[c]	Pos (weak)	Neg	Neg	Neg	Neg	Neg[c]	Pos (weak)	Neg	Neg
Phenotype 2	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Pos	Neg[c]	Pos	Pos	Neg
b) Fibroadenoma with sclerosing adenosis															
Phenotype	Pos	Neg	Pos	Neg[c]	Neg[c]	Neg[c]	nd[e]	Pos	Neg	Neg	Neg	Neg	nd[e]	nd[e]	Neg

a To facilitate comparisons the order of the markers is the same as in Table 2.
b See Fig. 10C.
c Less than 0.5% of the cells are positive.
d There are few cells with the phenotype CK15+/CK19+/Vim+.
e nd, not determined.

the other hand, were found contiguous to single layers of CK15+/CK19− cells. Cells in HUT 2 were CK15 positive, CK19 negative, CK8 positive, PgR positive, vimentin positive, Ki67 positive, Bcl2-positive, GATA-3 positive, and p53 negative (not shown; Table 2). About 5% of the cells were positive for Ki67 in both lesions, but none exhibited p53 mutations as judged by staining with the p53 antibody. It should be mentioned that we observed HUT1 lesions adjacent to ADH1; the phenotype of the latter is described below.

2.2.1.2.2. *ADHs*. Visual inspection of serial sections stained with CK15 and CK19 antibodies revealed two types of ADHs (ADH 1 and 2) that were CK15−/CK19+, but differed in the expression of vimentin (Table 2). Single cell layers with all four CK15/CK19 phenotypes (CK15+/CK19+, CK15+/CK19−, CK15−/CK19+, CK15−/CK19−), often of the columnar type, were found contiguous to ADH 1 (Fig. 8E,F; blue arrows; Panel B). Cells in the ADH were CK15 negative, expressed CK19 (red arrows in Fig. 8E,F), had attenuated levels of CK8, were ER and PgR negative, CD44 positive, vimentin negative, Ki67 positive, Bcl-2 negative, GATA-3 negative, and p53 positive (Fig. 8G; red arrow), just like the invasive cells (Table 2; see also Fig. 8H). Type 2 ADHs were much less abundant than the ADH 1 ones and as a result it was not possible to ascertain the phenotype of the adjacent non-malignant cells.

2.2.1.2.3. *DCIS and invasive cells*. The tumour contained multiple and closely located *in situ* elements of the Comedo type i.e. grade 3 (Silverstein et al., 1995). Panel C in Fig. 8, show representative pictures of DCIS stained with antibodies against CK15 (polyclonal) (I), CD44 (J), vimentin (K), Bcl-2 (L; note that only cells in the acinus are positive), and psoriasin (S100A7) (M). About 50% of the DCIS were vimentin positive and in some cases part of the DCIS was vimentin negative (red arrow in K). With very rare exceptions DCIS were negative for CK15. All DCIS had a high proliferation index, were negative for the anti-apoptotic protein Bcl-2, and expressed p53, psoriasin (S100A7), and MRP-14 (S100A9) just like the invasive cells. About 50% of the DCIS expressed vimentin, sometimes in a mosaic fashion (Table 2), suggesting that its expression is either lost at later stages of progression, or that the vimentin negative cells have a greater malignant potential.

2.3. *CK15 positive cells in non-malignant biopsies, large central collecting ducts of the nipple, and some benign conditions of the breast*

2.3.1. *Non-malignant biopsies*

IHC analysis of non-malignant tissue biopsies from 70 of the patients included in this study revealed the presence of CK15+ positive cells in 45 of the samples for which we had enough material for analysis. In most cases, cells expressing CK15 (Fig. 9A; red arrows) were negative for CK19 (Fig. 9B; red arrows) and vise versa, supporting the contention that the expression of these cytokeratins may be mutually exclusive under certain conditions. Only a few areas in 11 out of 70 of the samples analyzed showed expression of both CK15 and CK19 by the same cells (Fig. 9C,D; blue arrows) suggesting that only certain cell types may co-express both cytokeratins.

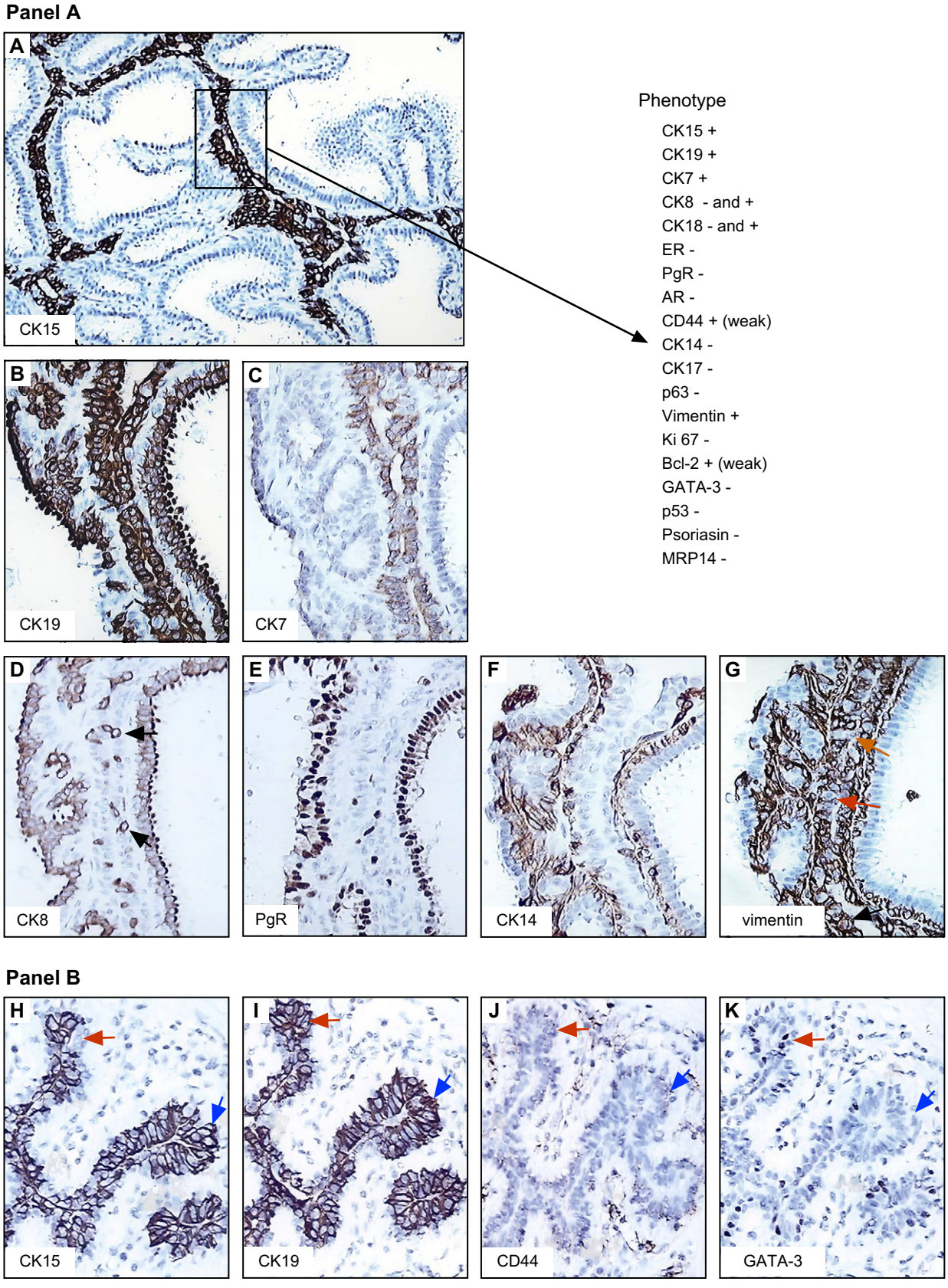


Figure 6 – Phenotype of non-malignant CK15+/CK19+ cells present in tumour 71. Panel A. Serial sections of paraffin-embedded tissue stained with antibodies against (A) CK15 (monoclonal), (B) CK19, (C) CK7, (D) CK8 (the black arrows indicate strongly positive cells), (E) (PgR), (F) CK14 and (G) vimentin. Only the area boxed in (A) is shown at larger magnification in figures B–G. The red arrows in G indicate vimentin negative cells. The phenotype of the cells is given at the top of Panel A. Panel B. Another area of the same section reacted with antibodies against CK15 (H), CK19 (I), CD44 (J), and GATA-3 (H). Blue arrows indicate CK15+/CK19+ cells, while the red arrows indicate CK15−/CK19+ cells which are differentiated as judged by the expression of GATA-3 (K).

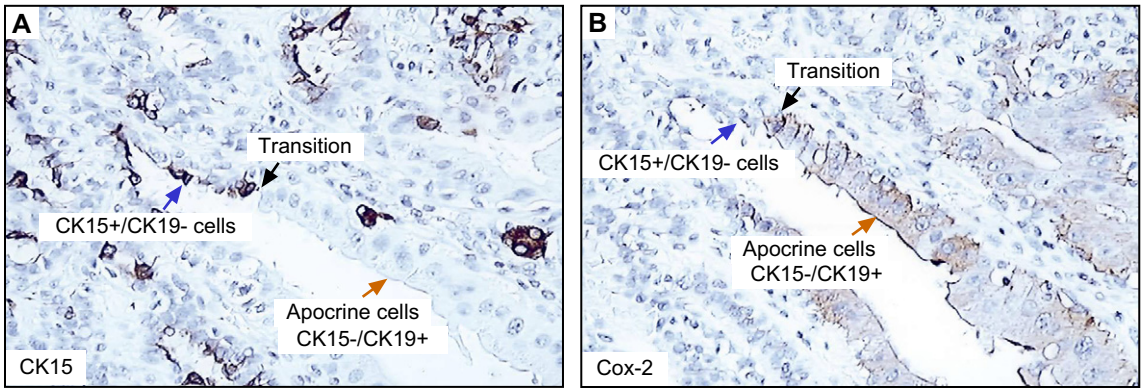


Figure 7 – CK15+/CK19+ columnar cells next to cells undergoing apocrine metaplasia. (A) and (B) serial sections of an area of tumour 71 with CK15+/CK19− columnar cells contiguous to cells undergoing apocrine metaplasia reacted with antibodies against CK15 (monoclonal) (A) and COX-2 (B) respectively. Apocrine cells are indicated with a red arrow. Non-apocrine cells are indicated with a blue arrow. The transition between CK15+/CK19− cells and apocrine cells, which are CK15−/CK19+, is indicated for reference.

Heterogeneous expression of CK19 in TDLUs where branching would be expected to occur during pregnancy has been reported (Bartek et al., 1990), but their relation to CK15 expression has not been assessed.

2.3.2. *Large central collecting ducts of the nipple*

We also had access to paraffin-embedded tissue collected from other parts of the mamma, like the nipple areola complex (NAC), for most of the patients included in this study (Table 1). Given that the collecting ducts participate in the formation of the developing breast (Fata et al., 2004; Russo and Russo, 2004) we surmised that these structures represented a potential source of progenitor cells. Accordingly, we analyzed the IHC staining patterns of the large central collecting ducts (Going and Moffat, 2004) of six of the patients that presented with tumours located distant to the nipple. As illustrated by the sample from patient 27, the results revealed abundant number of CK15 positive cells in these ducts (Fig. 10A; Panel A), which presented a convoluted epithelial profile. IHC analysis of consecutive serial sections allowed us to follow groups of cells that were positive for CK15+ and that exhibited the phenotype: CK19 positive (Fig. 10B), weak positive for CK8 (Fig. 10C; isolated cells staining strongly with the CK8 antibody were also observed and are indicated with a red arrow), ER, PgR (Fig. 10D), and AR negative, CD44 weakly positive, CK14 positive (Fig. 10E), CK17 negative, p63 negative, vimentin negative, Ki67 negative, Bcl-2 positive, GATA-3 negative (Fig. 10F), and p53 negative (Fig. 10, Panel A; Table 3). Given their phenotype the CK15+/CK19+/CK14+ cells have been termed collecting duct progenitors.

The existence of the CK15+/CK19+/CK14+ phenotype in single cells was confirmed by triple immunofluorescence analyses of formalin fixed paraffin embedded collecting ducts as shown in Panel A of Fig. 11. These studies also revealed CK15+/CK19+ cells that were CK14 negative as well as rare cells that were CK15+/CK19−/CK14− (Fig. 11, Panel A) or CK15−/CK19+/CK14+ (not shown). Similar studies using the vimentin antibody validated the IHC findings concerning the existence of CK15+/CK19+ cells that were vimentin negative and uncovered a much less abundant population of cells that were positive (Fig. 11, Panel B). The studies also exposed CK15+/CK14+ cells located in the luminal compartment that were negative for CK8 (Fig. 11, Panel C). No attempts were made at this time to extensively probe the cellular phenotype of the collecting ducts using IHC generated data as in some cases only few cells in the ducts expressed a given phenotype.

Careful analysis of sections of the large central collecting ducts revealed areas with hyperplasia in about 5% of the ducts. Cells in these areas stained strongly with the CK8 antibody (Fig. 10G; Panel B), were CK15 negative (Fig. 10H), CK19 positive, CD44 strong positive, ER, PgR and AR positive (Fig. 10I), Ki67 negative, vimentin negative, Bcl-2 negative, and GATA-3 positive (Fig. 10J; Table 3).

2.3.3. *Benign breast conditions*

Given the fact that CK15/CK19 positive luminal cells were first observed in benign hyperproliferative conditions (Celis et al., 2007), we analyzed by IHC samples from a few selected benign conditions that included a radial scar with adenosis, a fibroadenoma with sclerosing adenosis, and a case of juvenile fibroadenoma with ductal epithelial hyperplasia (Mies and Rosen, 1987). Besides revealing a few CK15+/CK19+ lesions in all three conditions, the results showed that in the juvenile fibroadenoma all the ducts in the sections exhibited a striking mosaic staining pattern with alternating CK15 positive (red arrows) and negative (blue arrows) areas (Panel A, Fig. 12A). As shown in Fig. 12A,B, CK15 positive areas stained weakly for CK8 (red arrows), while CK15 negative areas stained strongly (blue arrows). Red arrows in Panel B of Fig. 12 indicate CK15 positive areas in sections stained with antibodies against CK19 (Fig. 12C), PgR (Fig. 12D) and Bcl-2 (Fig. 12E). Lack of staining of the CK15 positive cells with the vimentin antibody was confirmed by triple immunofluorescence analysis (vimentin, CK8 and CK15) as shown in Fig. 13. The extended phenotype of the CK15 positive (phenotype 1) and negative (phenotype 2) cells is presented in Fig. 12A,B as well as in Table 3.

The IHC analysis of the fibroadenoma with sclerosing adenosis identified in addition a cellular phenotype that was CK15 positive, CK19 negative, CK14 positive, CK7 positive, CK8 positive, ER negative, PgR negative, AR negative and

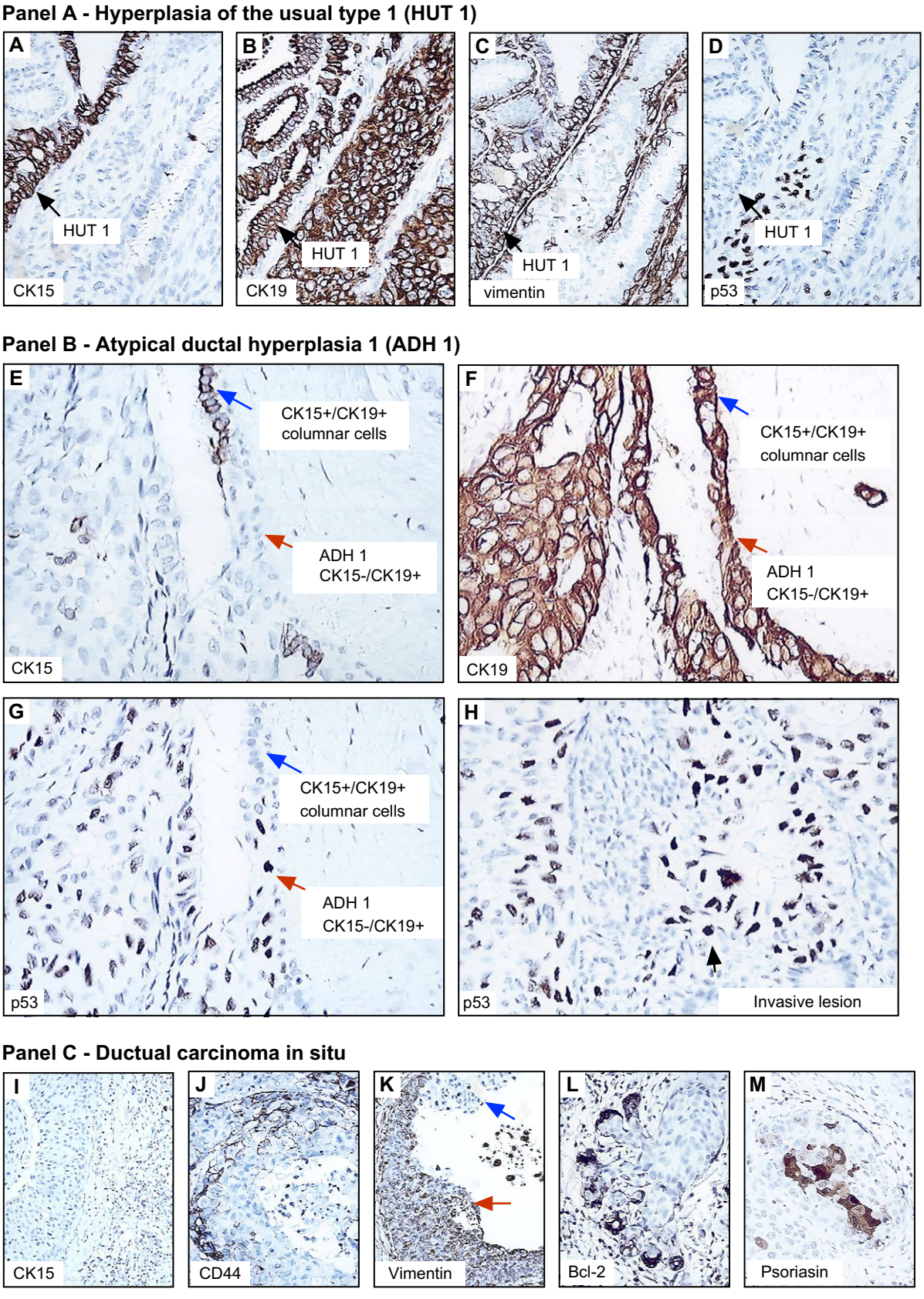


Figure 8 – IHC of non-malignant and premalignant lesions present in tumour 71. Panel A. Area with HUT 1 contiguous to CK15+/CK19+ cells stained with antibodies against CK15 (monoclonal) (A), CK19 (B), vimentin (C) and p53 (D). Panel B. ADH 1 contiguous to CK15+/CK19+ columnar cells reacted with antibodies against CK15 (E), CK19 (F), and p53 (G) (Table 2). For reference, (H) shows the invasive part of the tumour reacted with p53. Panel C. Ductal carcinoma *in situ* from T71 reacted with antibodies against CK15 (I), CD44 (J), vimentin (K), Bcl-2 (L), and psoriasin (S100 A7) (M). The red arrow in (K) indicates vimentin positive cells, while the blue one indicates negative ones.

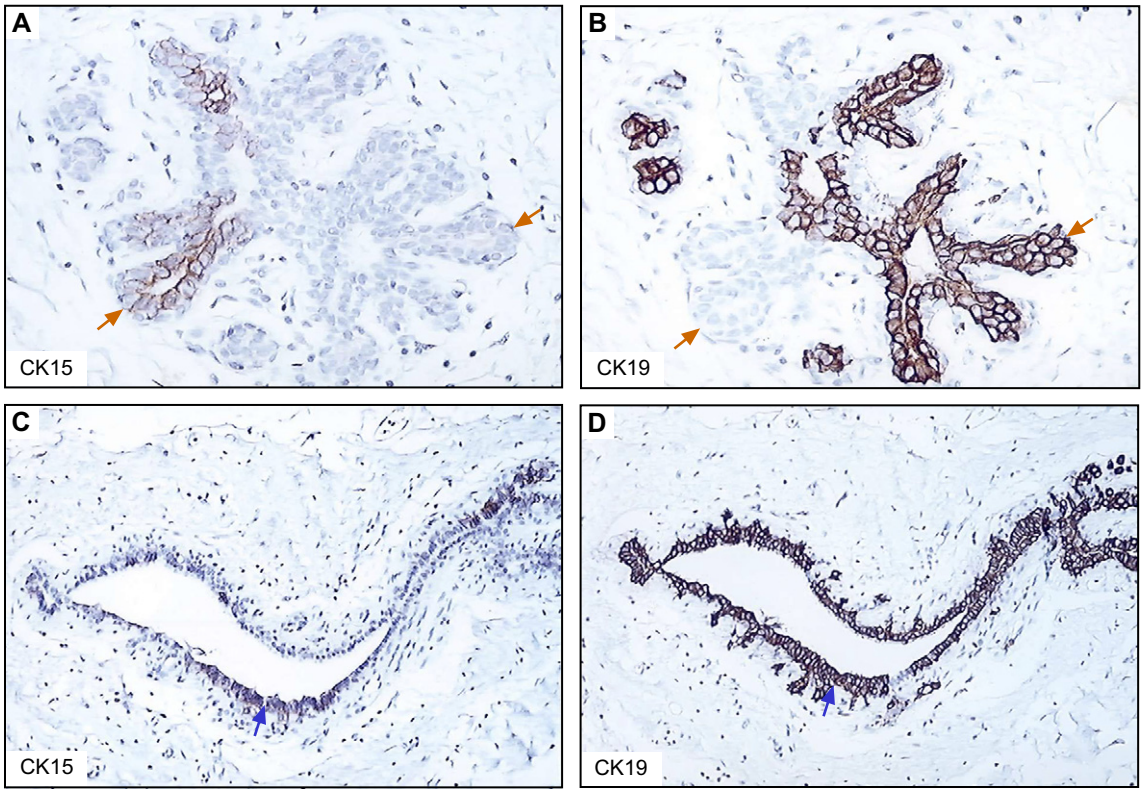


Figure 9 – CK15+ cells in normal TDLUs. Serial sections of normal ducts and lobules from biopsies of patients 14 (A, B) and 54 (C, D) stained with the CK15 (monoclonal) (A, C) and CK19 antibodies (B, D), respectively. Red arrows in (A) and (B) indicate cells that express either CK15 or CK19. Blue arrows in (C) and (D) indicate cells that co-express CK15 and CK19.

vimentin negative (results not shown; Table 3). Only a very small amount of tissue was available from the patient, a fact that limited the number of assays that could be performed.

3. Discussion

Breast cancer is a complex and heterogeneous disease and the histological parameters currently being used for patient stratification are clearly not powerful enough to distinguish the full range of lesions that can occur. Molecular profiling techniques, on the other hand, have provided a more comprehensive classification of breast carcinomas and today five different breast cancer subtypes representing biologically distinct disease entities have been identified based on cell type origin and differentiation as well as HER-2 receptor status: these include basal-like, luminal A, luminal B, normal breast tissue-like, and ERBB2 positive (Sorlie, 2004). Patients with a luminal ER positive phenotype have a relatively good prognosis as compared with basal-type tumours, which have a much shorter overall and disease-free survival period (Sorlie et al., 2003). Basal-like tumours, on the other hand, are characterised by being ER and PgR negative and by the expression of CK's 5 and 17 as well as cell cycle regulated genes (Ross et al., 2000; Whitfield et al., 2002; Sorlie et al., 2003; Livasy et al., 2006; Sorlie et al., 2006). Recently, Farmer and colleagues (Farmer et al., 2005) using principal component analysis and hierarchical clustering analysis of cDNA microarray data identified a novel subset of breast tumours with increased androgen signaling and apocrine expression profile that they termed "molecular apocrine", as these lesions do not exhibit all the histopathological features of classical apocrine carcinomas. Molecular apocrine tumours share some common expression characteristics with the ERBB2 class in the Stanford classification and exhibit some of the features of the basal group, underlining the heterogeneity of these lesions. Our own proteomic studies (Celis et al., 2006a,b) have shown that apocrine tumours correspond to a subtype of the basal-like carcinomas, and Bertucci and colleagues have recently presented evidence indicating that typical medullary breast carcinoma (MBC) are also part of the basal-like carcinoma spectrum (Bertucci et al., 2006; Vincent-Salomon et al., 2007), suggesting that the basal-like group may be more heterogeneous than previously thought. Indeed, the latter may also be the case for other molecular subtypes, as the Stanford classification is by no means exhaustive.

3.1. *CK15 positive carcinomas cut across subtypes as defined by histopathological or molecular parameters*

The results presented here identified a novel subset of breast carcinomas that express the epithelial stem cell marker CK15 (Jih et al., 1999; Ohyama et al., 2006; Figueira et al., 2007; Villadsen et al., 2007) and that comprised 5% of the high-risk lesions examined. The subset included carcinomas that were assessed by histological examination as being of the ductal

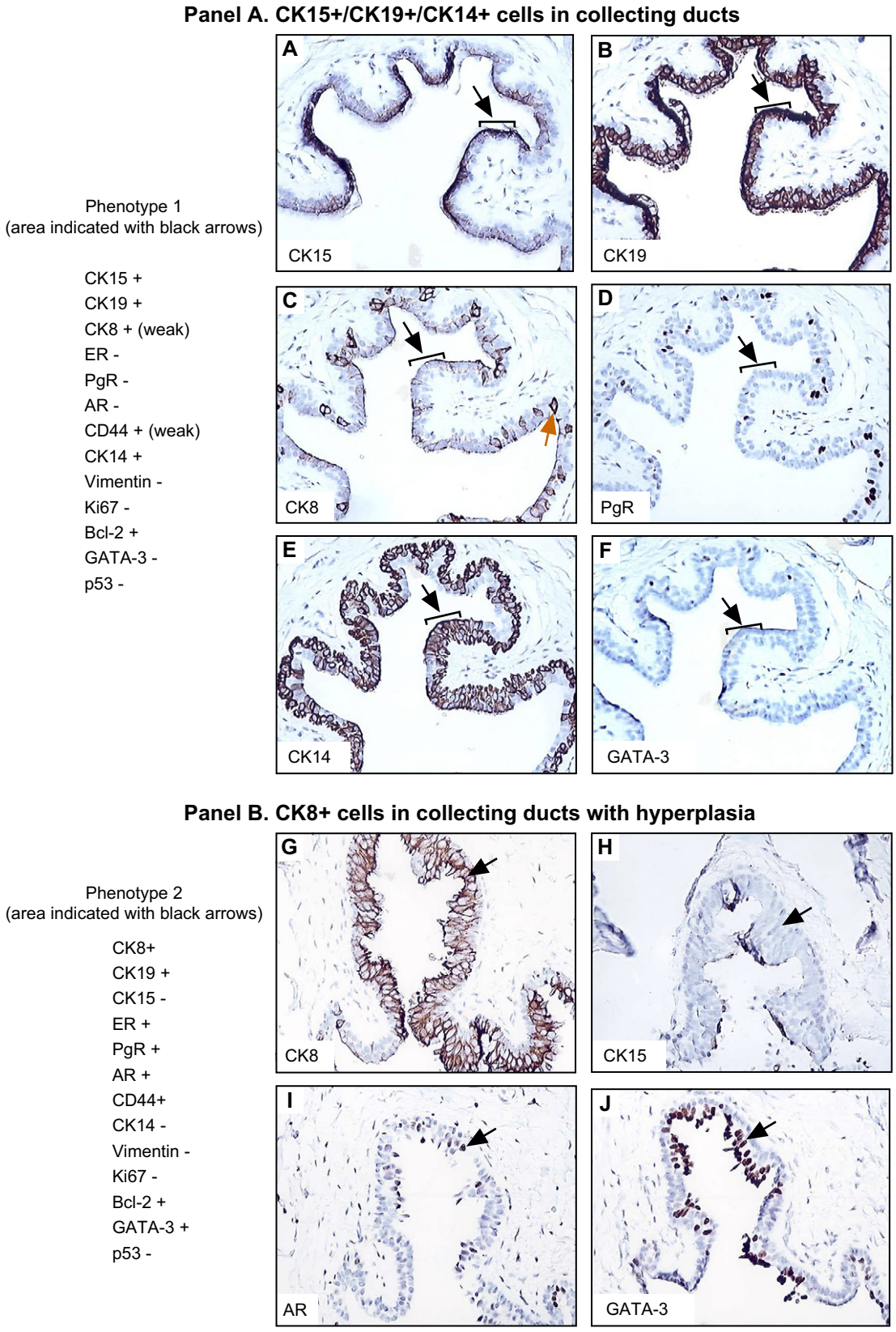


Figure 10 – CK15+/CK19+/CK14+ progenitor 1 cells in human large collecting ducts of the breast. Panel A. Serial sections of collecting ducts from patient 27 stained with antibodies against CK15 (polyclonal, Aviva) (A), CK19 (B), CK8 (C), PgR (D), CK14 (E) and GATA-3 (F). The phenotype of the progenitor cells (black arrows) in given for reference. The red arrow in (C) indicates a CK8 positive cell. Panel B. CK8 positive cells present in a large collecting duct of patient 27 with epithelial hyperplasia. Serial of collecting ducts were stained with antibodies against CK8 (G), CK15 (H), AR (I), and GATA-3 (J). The phenotype of the CK8 positive cells indicated with a black arrow is given for reference.

(tumours 23, 42, 57 and 88) and lobular types (tumours 65 and 66) (Table 1). Three of the ductal carcinomas, 23, 42, and 57 were ER and PgR negative; tumour 23 of basal-like type being Her-2 neu positive, while the fourth (T88) was ER, PgR and Her-2 neu positive and may correspond to a luminal B subtype. Both of the lobular tumours were ER/PgR positive and were E-cadherin negative as determined by 2D gel analysis, immunoblotting, and IHC. Interestingly, 5 out of 6 of the CK15 positive lesions presented with ALN metastases (Table 1), a predictor of poor prognosis (Nemoto et al., 1983; Rack et al., 2003). Clearly, the CK15 positive carcinomas identified in this study cut across subtypes defined either by classical histopathological or molecular parameters and raise questions about the classifications currently available to assess prognosis and therapeutic treatment. It should be emphasized that this is the first reported research effort using complementary proteomics and histological analyses of a large, well-matched, exhaustively characterized set of breast carcinomas. All tumors were sampled at several topological locations, and the various tissue biopsies were repeatedly analyzed by 2D PAGE and IHC performed on consecutive sections, to increase the likelihood of detecting rare cell populations present only in a very small proportion of the tumor. Efforts are currently underway to gather a large number of retrospective breast samples with long-term follow-up and clinical information in order to determine the prognostic value of CK15 in breast cancer.

3.2. *Double positive CK15/CK19 progenitor cells and their relation to pre-malignant lesions and invasive disease*

An important finding from this and a previous study (Celis et al., 2007) is the observation that expression of CK15 and CK19 may be mutually exclusive under certain conditions. Indeed, IHC analysis of non-malignant biopsies indicated that most cells expressing CK15 were negative for CK19 and vise versa. Only a few areas in 11 out of 70 of the samples analyzed showed expression of both CK15 and CK19 by the same cells suggesting that only certain cell types co-express both cytokeratins. In line with these observations, we found only one tumour, a lobular lesion (patient 65) that expressed both CK15 and CK19 in the same cells, although the tumour area also exhibited cells that expressed either one of these cytokeratins. Moreover, cells in apparently normal ducts close to the tumour cells expressed all three phenotypes (CK15+/CK19+, CK15+/CK19−, CK15−/CK19+). This striking finding suggested that different precursor cells may have contributed to the intratumour heterogeneity displayed by this lesion. Indeed, this was supported by the study of tumour 71, a CK15−/CK19+/p53+ carcinoma that contained non-malignant epithelial cells that exhibited a variety of p53 negative, CK15/CK19 cellular phenotypes (CK15+/CK19+; CK15+/CK19−; CK15−/CK19+; CK15−/CK19−), often associated with simple columnar cells (Simpson et al., 2005; Pinder and Reis-filho, 2006; Dabbs et al., 2006). The CK15+/CK19+ precursor(s) is perhaps the most primitive as the cells did not express ER or PgR, the great majority were vimentin positive, and were negative for Ki67 and GATA-3, the latter a transcription factor that regulates luminal differentiation (Kouros-Mehr et al., 2006; Asselin-Labat et al., 2007; Sutherland et al., 2007) (Table 2). Cells exhibiting all four of the cellular CK15/CK19 phenotypes were observed contiguous to areas with ADH 1 which contained cells that were p53 positive, ER and PgR negative, vimentin negative, and psoriasin positive (S100A7), just like the CIS and the invasive cells (Table 2). In a few areas we also observed ADHs with cells that were p53 positive, vimentin positive, and CK15 negative (ADH 2; Table 2), but these lesions were less abundant than the vimentin negative ones and as a result it was not possible to ascertain the phenotype of the adjacent non-malignant cells. As a whole, the results are taken to imply that cells with all four phenotypes may drive progression towards ADH following p53 mutation (Bartek et al., 1990; Clahsen et al., 1998; Rohan et al., 2006; Lacroix, 2006; Herceg and Hainaut, 2007), and perhaps other aberrations and epigenetic changes, leading to development of cells that either lost CK15 or gained CK19 expression (Fig. 14). Most likely, cells in the ADHs accumulated additional mutations and/or epigenetic changes over time enabling them to progress to DCIS and invasive disease. At present we do not know what the relation is between the various precursors we have identified; although we speculate that the CK15+/CK19+ cells, which are the most dedifferentiated and progenitor-like, may give rise to the more differentiated cellular phenotypes (Table 2; Fig. 14), thus generating the daunting heterogeneity displayed by this lesion. A precise answer, however, will require an in depth analysis of additional samples as well as knowledge pertaining to the role of the tumour microenvironment on phenotype selection (McCawley and Matrisian, 2001; Wiseman and Werb, 2002). Molecular heterogeneity of breast tumours has also recently been described by Shipitsin et al. (2007), who found that CD44+ and CD24+ cells purified from individual breast carcinomas were clonally related, but not identical. The results were taken to imply that the tumour cells originate from a stem-like progenitor cell, which then diverts genetically to generate the heterogeneity observed in the tumours.

Our studies also provided evidence for the plasticity of the precursors as CK15+/CK19− cells could be seen progressing towards pre-malignant lesions as well as towards the formation of benign apocrine metaplasia (Fig. 14).

3.3. *CK15/CK19/CK14 triple positive progenitor cells in the large collecting ducts of the breast*

Considering that the collecting ducts participate in the formation of the developing breast (Fata et al., 2004; Russo and Russo, 2004) and that lobules and large ducts of normal breast tissue may be derived from the same stem cell (Tsai et al., 1996) we inferred that these structures represented a potential source of progenitor cells. Indeed IHC analysis of these ducts revealed dedifferentiated progenitor-like cells (collecting duct progenitor) with a similar phenotype to that of the CK15+/CK19+ progenitor identified in T71, except that they also expressed CK14 and were vimentin negative (Fig. 14). We also observed a minor population of CK15+/CK19+/vimentin+ cells in the ducts that may represent an even more primitive precursor if one considers their relative low abundance. Loss of CK14 expression by the collecting duct progenitor, and loss or gain of other markers most likely generates the CK15+/CK19+/CK14− luminal restricted progenitor

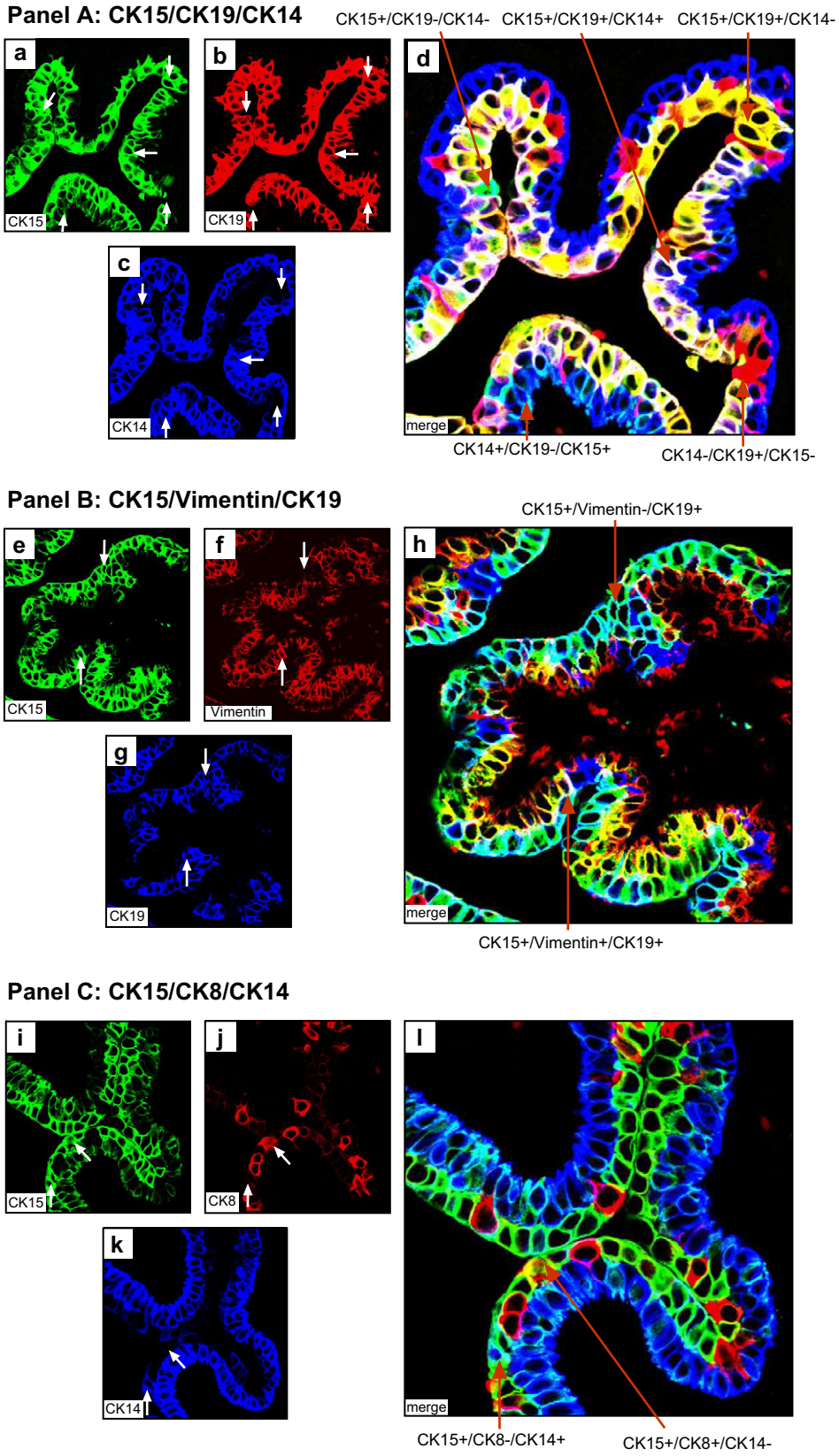
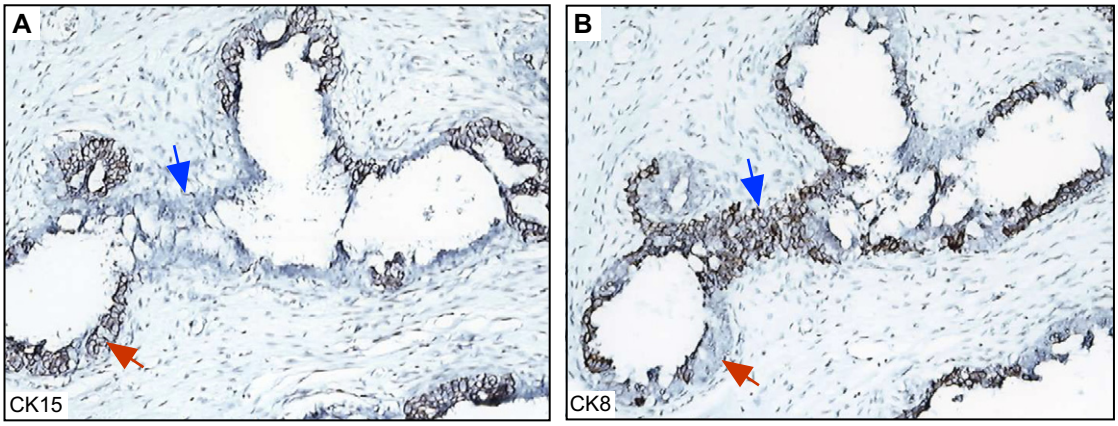


Figure 11 – Indirect triple-label immunofluorescence analysis of paraffin-embedded nipple tissue sections from patient 27. (A) Tissue section reacted with antibodies against CK15 (Alexa Fluor® 488; green channel, subpanel a), CK19 (Alexa Fluor® 594; red channel, subpanel b), and CK14 (Alexa Fluor® 633; blue channel, subpanel c), (B) Tissue section reacted with antibodies against CK15 (Alexa Fluor® 488; green channel, subpanel e), vimentin (Alexa Fluor® 594; red channel, subpanel f), and CK19 (Alexa Fluor® 633; blue channel, subpanel g). (C) Tissue section reacted with antibodies against CK15 (Alexa Fluor® 488; green channel, subpanel i), CK8 (Alexa Fluor® 594; red channel, subpanel j) and CK14 (Alexa Fluor®

Juvenile fibroadenoma

Phenotype of areas indicated by red arrows	Phenotype of areas indicated by blue arrows
CK15 +	CK15 -
CK19 +	CK19 +
CK8+ (weak)	CK8 - +
ER -	ER +
PgR -	PgR +
AR -	AR +
CD44 + (weak)	CD44 +
CK14 -	CK14 -
Vimentin -	Vimentin +
Bcl-2 + (weak)	Bcl-2 +
GATA-3 -	GATA-3 +
p53 -	p53 -

Panel A



Panel B

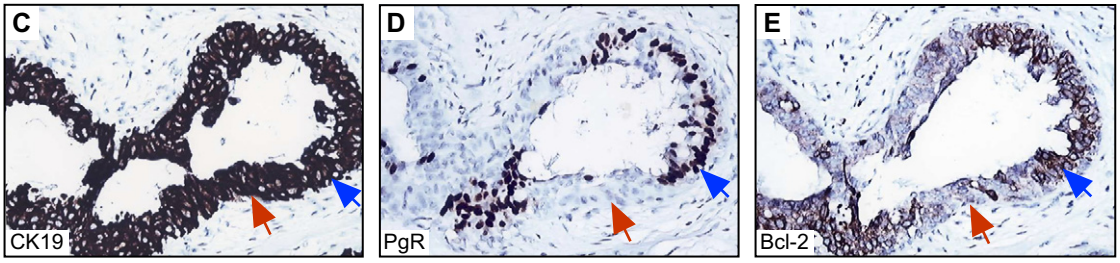


Figure 12 – CK15+/CK19+ cells present in the ducts of a juvenile fibroadenoma with epithelial hyperplasia. Panel A. Serial sections stained with the CK15 (polyclonal Aviva) (A) and CK8 antibodies (B) respectively. Red arrows indicate areas of the duct with CK15 positive cells, while blue ones indicated CK8 positive cells. Panel B. Serial sections of another area of the juvenile fibroadenoma stained with antibodies against CK19 (C), PgR (D), and Bcl-2 (E). Red arrows indicate areas of the duct with CK15+/CK8− cells, while blue ones indicate CK8+/CK15− cells. The extended phenotype of both cell types are given for reference.

cells, which were also observed in the collecting ducts (Fig. 10, Panel A; Fig. 14). Triple immunofluorescence studies also revealed CK15+/CK19−/CK14+ cells, some of which located to the basal layer (Fig. 11d, Panel A), and that may correspond to a lineage-restricted myoepithelial progenitor generated by loss of CK19 and possibly loss and gain of other markers (Deugnier et al., 2002; Gudjonsson et al., 2005; Adriance et al., 2005) (Fig. 14). Expression of CK15 by the myoepithelial lineage seems to be retained until late stages of differentiation as we have previously published evidence for the existence of

633; blue channel, subpanel k). Cells with different phenotypes are indicated with white arrows in the different subpanels. In the merged images (subpanels d, h, and l, respectively), cells expressing different combinations of antigens are indicated with arrows and their phenotype described in a caption. In J, Panel C, a single cell luminal cell with the phenotype CK15+/CK8+/CK14− is observed. These cells, however, are more common in other areas of the sections, although still much less abundant than the CK15+/CK8+/CK14− ones.

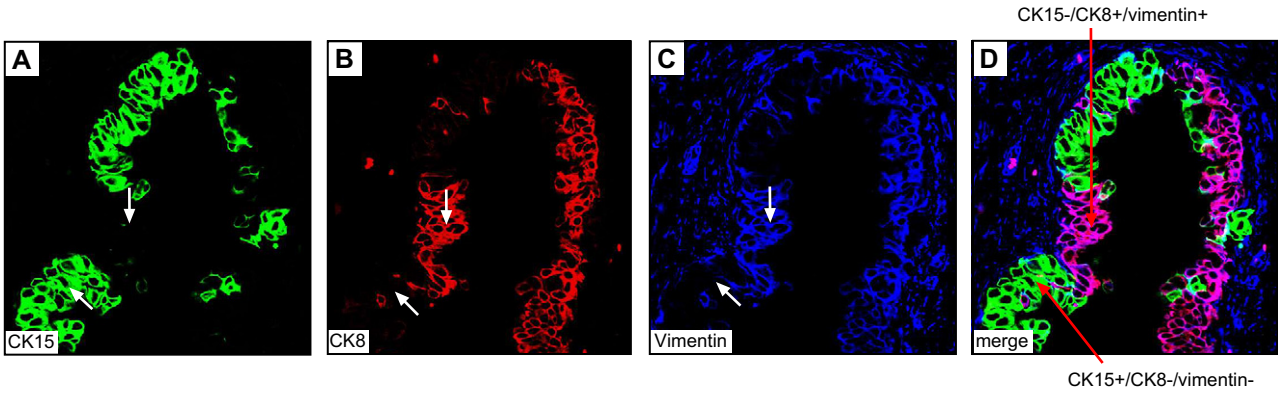


Figure 13 – Indirect triple-label immunofluorescence analysis of paraffin-embedded tissue sections from a patient with juvenile fibroadenoma. A tissue section was stained with antibodies against CK15 (Alexa Fluor® 488; green channel, subpanel A), CK8 (Alexa Fluor® 594; red channel, subpanel B), and vimentin (Alexa Fluor® 633; blue channel, subpanel C). Cells with different phenotypes are indicated with white arrows in the different subpanels. In the merged image (subpanel D), two different cell populations expressing different combinations of antigens are indicated with arrows and their phenotype described in a caption.

CK15+/p63+ putative lineage restricted myoepithelial precursors (Celis et al., 2007). At present, we do not know at what stage during myoepithelial differentiation CK15 is lost.

It is well-known that carcinomas of the breast arise as a result of mutations and/or epigenetic changes in cells originating in the TDLU's rather than in the large ducts (Ohuchi et al., 1984; Russo and Russo, 2004), a fact that implies that the collecting duct progenitor cells may be refractory to carcinogenesis. Indeed, only benign adenomas of the nipple have ever been reported, and these are very rare (Sugai et al., 2002). Work by Russo and coworkers, on the other hand, have presented evidence for the protective effect of pregnancy on cancer development (Russo et al., 2005 and references within). Their studies suggest that putative stem cells 1, which are the targets of neoplastic events, shift towards stem cells 2 during parity leading to differentiation of the mammary gland, a process that is believed makes the gland refractory to carcinogenesis. Even though the authors have found differences in the molecular signature of the stem 1 and 2 cells, it is unclear what the molecular mechanisms that underlie the phenomenon are (Russo et al., 2005). Further analysis of the collective duct progenitor cells, in particular the proteomic characterization of their phenotype along the ductal lobular tree using microdissection techniques, should contribute to a better understanding of normal mammary gland development and breast carcinogenesis.

3.4. *Relationship between the phenotypes of the precursor cells and adult breast stem cells*

Recent work by Villadsen et al. (2007) has identified a putative stem cell niche in human ducts as well as in zones containing progenitor cells in lobes. Putative stem cells residing in ducts were shown to be positive for CK14 and CK19 and enriched in CK15 and the stage-specific embryonal antigen-4 (SSE-4) (Asselin-Labat et al., 2006). Studies of Clarke and colleagues, on the other hand, have indicated that normal breast stem cells may be ER/PgR negative in line with studies performed in mice (Clarke, 2005). Based on these data it would seem reasonable to assume that a likely phenotype for normal adult breast stem cells is CK15+/CK19+/CK14+/ER−/PgR−, even though the co-existence of all three keratins in the same cell has not yet been proved (Fig. 14). As indicated in Fig. 14, these cells give rise to lineage restricted precursors that in turn generate luminal (ductal and alveolar) and myoepithelial cells. As shown in the case of T71, the lineage restricted luminal progenitor may be target for mutations and epigenetic changes that lead to cancer development (Fig. 14).

Interestingly, the IHC analysis of the 120 carcinomas revealed one tumour (T16, Table 1) that was CK15 negative, but co-expressed CK's 14 and 19 (data not shown). This high-grade carcinoma was p53 positive and corresponded to a triple negative lesion (ER−/PgR−/Her-2 neu −). The phenotype of the malignant cells in this lesion (CK15−/CK19+/CK14+/ER−/PgR−) makes it likely that this carcinoma arose by direct malignant transformation of the breast stem cells or closely related progenitor cells, loosing CK15 expression in the process, as presented in the tentative diagram for the evolution of breast cancer phenotypes shown in Fig. 14. At present we cannot exclude the occurrence of triple CK15+/CK14+/CK19+ lesions (or other combinations of these markers), but have no evidence at this stage to support their existence.

The relationship between the adult stem cells and the progenitor cells identified in the collecting ducts is at present unknown, although they may differ in their response to carcinogens. Also, their interactions with stromal cells and extra cellular matrix may be important in defining their biological properties (Fata et al., 2004).

3.5. *Conclusions*

Taken as a whole our studies raise the interesting possibility that CK15 may be a neutral cytokeratin whose cellular expression is permissive in progenitor cells that express multiple lineage-specific keratins. As these cells differentiate towards a specific lineage they become committed and go on to express only the phenotypic markers characteristic for that given lineage, at which time CK15 is no longer required at least

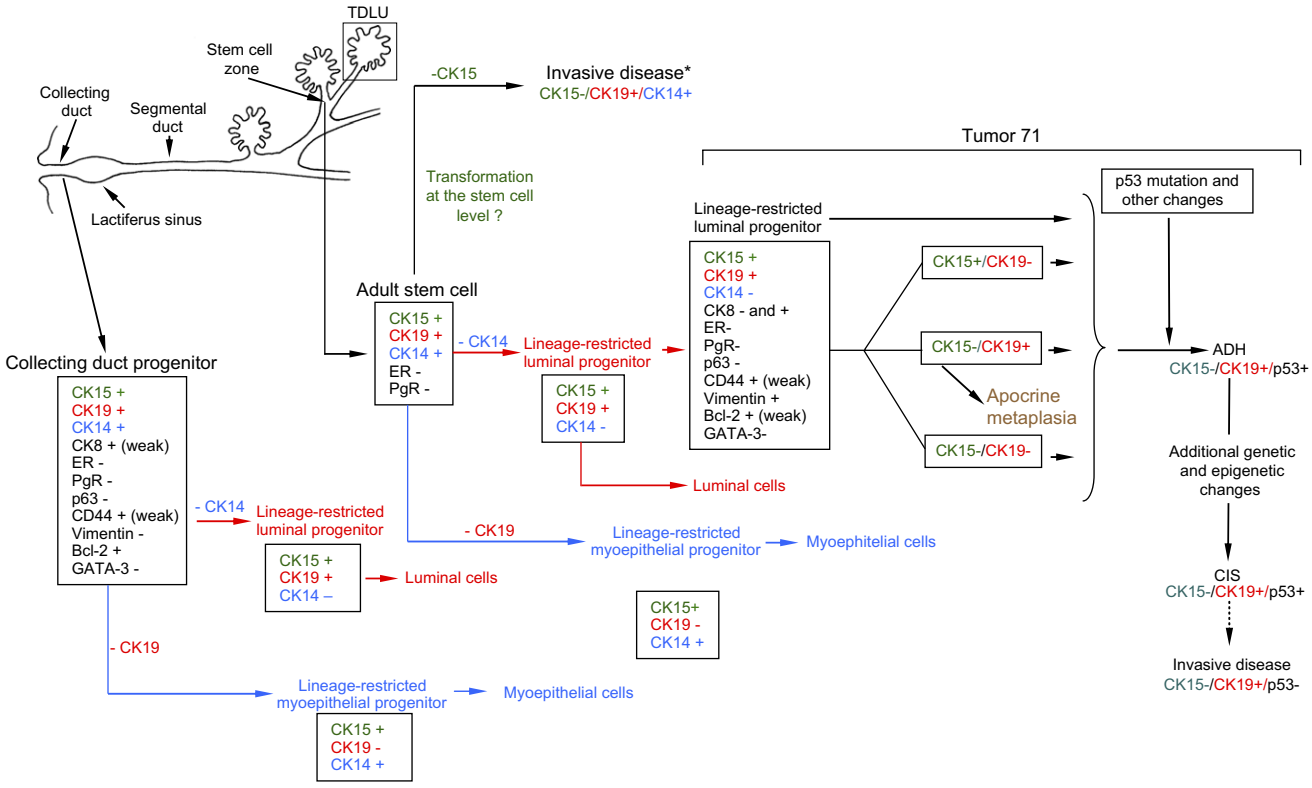


Figure 14 – Tentative diagram for the evolution of normal and cancer breast phenotypes. The nomenclature of the mammary ductal system is according to Wellings et al. (1975). The following structures are indicated starting from the nipple opening: collecting ducts, lactiferous sinus, segmental ducts, and terminal duct lobular unit (TDLU). The stem cell zone is found in the TDLU. The putative normal adult human breast stem cell phenotype was inferred from published data (Villadsen et al., 2007; Clarke, 2005). Stems cells have the capacity of self-renewal generating a copy of itself and a progenitor/amplifying cell that give raise to lineage-restricted precursors that in turn produce luminal (ductal and alveolar) and myoepithelial cells. As shown in the case of T71, the lineage restricted luminal progenitor may be target for mutations and epigenetic changes that lead to cancer development. Most of the cells in the lineage restricted progenitor in T71 are vimentin positive, although negative cells have also been observed. At present we do not know what the relation is between the various precursors we have identified; although we speculate that the CK15+/CK19+ cells, which are the most dedifferentiated and progenitor-like, may give rise to the more differentiated cellular phenotypes, thus generating the daunting heterogeneity displayed by this lesion. Cells with a similar phenotype to the CK15+/CK19+ precursors were also found in a juvenile fibroadenoma with epithelial hyperplasia. The relationship between the adult stem cells and the progenitor cells identified in the collecting ducts is at present unknown, although they may differ in their response to carcinogens. In the collecting ducts, most progenitor cells are vimentin negative, but there are a few cells that are CK15+/CK19+/vimentin+. Finally, we indicate in this tentative diagram for the evolution of breast cancer phenotypes that direct mutation(s) of the stem cells can give rise to CK15−/CK19+/CK14+ carcinomas as found for T16. *At present we cannot exclude the occurrence of triple CK15+/CK14+/CK19+ lesions (or other combinations of these markers), but have no evidence at this stage to support their existence.

at the same levels. Thus, the expression of CK15 alone or in combination with CK19 may be only compatible with the gene expression programme of some normal breast precursors and malignant carcinomas as shown in this study.

Currently, we are in the process of carrying out a comprehensive and systematic study of benign and pre-malignant lesions such as CIS in an effort to assess the biological potential of the progenitor cells we have identified and to correlate the data with the phenotype of breast cancers, in particular those that have been associated with the basal-like phenotype, apocrine tumours included, as both T65 and T71 exhibited extensive apocrine metaplasia. The ultimate aim of these studies is to map the whole possible range of precursor cell phenotypes that can occur and to gain a better understanding of the fate(s) of these cells when in a given biological and pharmacological setting. Towards this aim we have started a systematic proteomic analysis of established breast cell lines, in an effort to identify cell types that express similar phenotypes to those displayed by the putative progenitor cells identified in this study. By silencing single components at a time it should be possible to manipulate the fate of these cells, thus identifying some of the molecular mechanisms underlying malignant breast disease. In addition, identification of the cellular counterparts of the progenitor cells identified in this study in other organisms (e.g. mouse) where models of carcinogenesis and tumor progression are available should allow us to dissect the biological mechanisms that link the progenitor cells we identified to invasive disease. Unraveling the molecular pathways and mechanisms of malignant development for all precursor cell phenotypes will not only identify possible

therapeutic intervention points, but it would also lead to the development of therapies that selectively target the progenitor cells found in a patient's tumour.

An important implication of our studies relates to the clinical application of high-throughput molecular diagnostics/prognostics methodologies. The plasticity and phenotypic complexity of the cells we highlighted in this report cannot be ascertained by high-throughput methods as result of the latter's intrinsic averaging nature. Minor subpopulations of cells of high malignant potential present within a large tumor will escape detection by averaging methods, detracting from the predictive power of such approaches.

4. Experimental procedures

4.1. *Sample collection and handling*

Tissue biopsies from clinical high-risk patients (http://www.dbcg.dk) that underwent mastectomy were collected from the Pathology Department at the Copenhagen University Hospital 30–45 min after surgery. Samples for gel analysis were placed in liquid nitrogen and were rapidly transported to the Institute of Cancer Biology where they were stored at −80 °C. On average, a total of 45 min to 1 h elapsed between surgical sample acquisition and sample preparation. A complete list of carcinomas analyzed in this study is given in Table 1. We also had access to paraffin-embedded tissue blocs from nipple tissue as well as selected benign conditions (radial scar, juvenile fibroadenoma with epithelial hyperplasia, and a fibroadenoma with sclerosing adenosis). The project was approved by the Scientific and Ethical Committee of the Copenhagen and Frederiksberg Municipalities (KF 01–069/03).

4.2. *Two-dimensional gel electrophoresis and Western Immunoblotting*

Two dimensional polyacrylamide gel electrophoresis (isoelectric focussing (IEF)) was performed as previously described (O'Farrell, 1975; Celis et al., 2005b). Twenty to thirty, six-μm cryostat sections of frozen tissues were resuspended in 0.1 ml lysis solution (O'Farrell, 1975) and were kept at −20 °C until used (Celis et al., 2005a). A total of 40 μl were applied to the gels. The first and last sections of each sample were used for immunofluorescence analysis using cytokeratin 19 (CK19) antibodies as this epithelial marker is ubiquitously expressed by mammary epithelial cells (Moll and Moll, 1998). The availability of these pictures greatly facilitated the interpretation of the gel data as it gave a rough estimate of the ratio of glands/tumour cells to stromal tissue. 2D gels were analysed using the PDQUEST software from BioRad (version 7.3). Silver staining, compatible with mass spectrometry, was performed according to published procedures (Gromova and Celis, 2006). 2D gel Western immunoblotting was performed as previously described (Celis et al., 2006c).

4.3. *Protein Identification by mass spectrometry*

Protein spots were excised from dry gels and the gel pieces were re-hydrated in water. Gel pieces were detached from the cellophane film and cut into about 1 mm pieces followed by proteins "in-gel" digestion as previously described (Shevchenko et al., 1996). Samples were prepared for analysis by applying 2 μl of digested and extracted peptides on the surface of a 400/384 AnchorChip target (Bruker Daltonik, GmbH), followed by co-crystallization with α-cyano matrix. Mass spectrometry was performed using a Reflex IV MALDI-TOF mass spectrometer equipped with a Scout 384 ion source. All spectra were obtained in positive reflector mode with delayed extraction, using an accelerating voltage of 28 kV. The resulting mass spectra were internally calibrated using the auto-digested tryptic mass values visible in all spectra. Calibrated spectra were processed by the Xmass 5.1.1 and BioTools 2.1 software packages (Bruker Daltonik, GmbH). No restriction on the protein molecular mass and taxonomy was applied. The sequence database used for search was the NCBInr Database (comprehensive, non-identical protein database). A number of fixed (acrylamide modified cystein, i.e. propionamide/carbamidomethylation) and variable modifications (methionine oxidation and protein N-terminus acetylation) were included in the search parameters. The peptide tolerance did not exceed 50 ppm and as a maximum only one trypsin missed cleavage was allowed. The protein identifications were considered to be confident when the protein score of the hit exceeded the threshold significance score of 70 ($p < 0.05$) and nor less than 6 peptides were recognized. Whenever the protein score hit was close to the threshold significance score of 70, the Post Source Decay (PSD) was performed as an additional mean to confirm the identity of the proteins identified by peptide fingerprinting. The following PSD search parameters were used: peptide tolerance 50 ppm and MS/MS tolerance 1 Da without any restriction on the protein molecular mass and taxonomy.

4.4. *Antibodies*

The anti-peptide antibodies against MRP-14 (S100A9) (EP 010100) was prepared by Eurogentec (Liege, Belgium). Monoclonal antibodies against p53 (clone DO-7), p63 (clone 4A4), androgen receptor (AR; clone AR441), estrogen receptor (ER; clone 1D5), progesterone receptor (clone PgR636), COX-2 (CX-294), BCL-2 (clone 124), vimentin (clone Vg), E-cadherin (clone NCH-38), and Ki67 (clone MIB-1) were purchased from DakoCytomation (Glostrup, Denmark). Monoclonal antibodies recognizing CK's 14 (clone LL002), 15 (clone LHK15), CK17 (clone E3), and 19 (clone A53-B/A2.26) were from NeoMarkers (LABVISION, CA). A rabbit anti-peptide antibody against human CK15 was obtained from Aviva Systems Biology. The monoclonal antibody against CK18 was obtained from Cappel (Organon Teknika, ICN). The monoclonal antibody against GATA-3 was purchased from Santa Cruz Biotechnology Inc. The monoclonal antibodies against CK's 7 (clone RCK105) and 8 (clone M20) were purchased from MP Biomedicals (Irvine, CA). The monoclonal antibody against psoriasin (S100-A7) has been previously described (Ostergaard et al., 1999). The specificity of most of the antibodies used in this work was determined by 2-D PAGE immunoblotting (Celis et al., 2007).

4.5. *Immunohistochemistry (IHC)*

Following surgery, fresh tissue blocks were immediately placed in formalin fixative and paraffin embedded for archival use. Six-μm sections were cut from the tissue blocks and mounted on Super Frost Plus slides (Menzel-Gläser, Braunschweig, Germany), baked at 60 °C for 60 min, deparaffinised, and rehydrated through graded alcohol rinses (Moreira et al., 2005). Heat induced antigen retrieval was performed by immersing slides in 10 mM citrate buffer (pH 6.0) and microwaving in a 750 W microwave oven for 10 min. The slides were then cooled at room temperature for 20 min and rinsed abundantly in tap water. Non-specific staining of slides was blocked (10% normal goat serum in PBS buffer) for 15 min, and endogenous peroxidase activity quenched using 0.3% H_2O_2 in methanol for 30 min. Antigen was detected with a relevant primary antibody, followed by a suitable secondary antibody conjugated to a peroxidase complex (HRP conjugated goat anti-rabbit or anti-mouse antibody; DakoCytomation (Glostrup, Denmark). Finally, colour development was done with 3,3′-diaminobenzidine (Pierce, IL, USA) as a chromogen to detect bound antibody complex. Slides were counterstained with hematoxylin. Standardization of the dilution, incubation, and development times appropriate for each antibody allowed an accurate comparison of expression levels in all cases. At least three independent staining of the samples were performed for each antibody. Sections were imaged using either a standard bright field microscope (Leica DMRB) equipped with a high-resolution digital camera (Leica DC500), or a motorized digital microscope (Leica DM6000B) controlled by Objective Imaging's Surveyor Software (Objective Imaging ltd, UK) for automated scanning and imaging which enables tiled mosaic image creation. Original magnification for all images is 200×.

4.6. *Immunofluorescence on paraffin sections*

Fresh tumours were placed in formalin fixative and paraffin-embedded for archival use. Five-μm sections were cut from paraffin blocks of breast tissue samples mounted on Super Frost Plus slides (Menzel-Gläser, Braunschweig, Germany), baked at 60 °C for 60 min, deparaffinised, and rehydrated through graded alcohol rinses. Heat induced antigen retrieval as well as additional steps were carried out as described above. Antigens were detected by overnight incubation at 4 °C with primary antibodies at the appropriate dilution conjugated to Alexa Fluor® 488, Alexa Fluor® 594, and Alexa Fluor® 633 (Molecular Probes, OR, USA) or counterstained with TO-PRO-3. Sections were imaged using confocal laser scanning microscopy (Zeiss 510LSM).

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