Distribution and significance of nerve growth factor receptor (NGFR/p75^NTR) in normal, benign and malignant breast tissue

Jorge S Reis-Filho[1,2,*], Dawn Steele[1,*], Silvana Di Palma[3,*], Robin L Jones[1,4], Kay Savage[1], Michelle James[1], Fernanda Milanezi[2], Fernando C Schmitt[2] and Alan Ashworth[1]

[1]The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, UK; [2]IPATIMUP—Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal; [3]Royal Surrey County Hospital, University of Surrey, Guildford, UK and [4]Breast Unit, Royal Marsden Hospital, London, UK

Nerve growth factor receptor (NGFR) is a transmembrane glycoprotein without intrinsic tyrosine kinase activity, whose expression is not restricted to neural cells. NGFR is reported to act as a tumour suppressor, negatively regulating cell growth and proliferation. NGFR expression was immunohistochemically analysed in normal breast tissue and in 140 benign, biphasic and preinvasive breast lesions, in 22 tumours with myoepithelial differentiation and in two cohorts of breast cancer patients: a series of 245 invasive breast carcinomas studied with tissue microarrays and 37 high-grade invasive ductal carcinomas with basal-like immunophenotype. NGFR consistently displayed membrane reactivity in myoepithelial cells arranged as a continuous layer around normal ducts and lobular units, intralobular fibroblasts, vascular adventitia and nerve bundles. Myoepithelial cells of benign proliferations and pre-invasive lesions were consistently positive for NGFR. Scattered NGFR-positive cells were observed in solid areas of six out of nine cases of hyperplasia of usual type, whereas in flat atypia, lobular carcinoma *in situ* and virtually all cases of ductal carcinoma *in situ* (97.5%), NGFR was restricted to the myoepithelial layer. Positivity for NGFR was observed in 11 out of 245 (4.5%) breast carcinomas, nine out of 20 (45%) metaplastic breast carcinomas and 14 out of 37 (38%) basal-like breast carcinomas. NGFR expression in invasive tumours significantly correlated with that of cytokeratins 5/6 (*P*<0.05), 14 (*P*<0.0001) and 17 (*P*<0.0005) and EGFR (*P*<0.0001) and displayed an inverse correlation with oestrogen and progesterone receptors (both, *P*<0.0001). NGFR showed a statistically significant association with longer disease-free (*P*<0.05) and overall survival (*P*<0.01) in the cohort of patients with basal-like carcinomas. This study demonstrates the usefulness of NGFR as a new adjunct marker to identify myoepithelial cells in preinvasive lesions and myoepithelial differentiation in breast carcinomas. Furthermore, provisional data in a small number of basal-like breast carcinomas suggest that NGFR may identify a subgroup of basal-like breast carcinomas with good prognosis.
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Human p75^NTR, also known as nerve growth factor receptor (NGFR), maps to 17q21[1] and encodes a 75 kDa cell surface receptor glycoprotein that binds with similar affinity to the neurotrophin family of growth factors.[2–4] It is now apparent that expression of NGFR is ubiquitous and not limited to the nervous system,[4] being expressed in mature non-neural cells such as perivascular cells, dental pulp cells, lymphoidal follicular dendritic cells, basal epithelium of oral mucosa and hair follicles, prostate basal cells and myoepithelial cells.[4–6] Unlike the high-affinity nerve growth factor tyrosine kinase receptors (TrkA, TrkB and TrkC), NGFR has no intrinsic tyrosine kinase activity.[4] Studies in prostate and urothelial cancer suggest that NGFR may act as a tumour suppressor, negatively regulating cell growth and proliferation.[7–13]

Previous studies on NGFR expression in breast carcinomas showed no direct association between the expression of this receptor and either disease-free or overall survival.[14] However, the ratio between NGF:NGFR was reported to be of prognostic significance.[15] As the role of NGFR in breast cancer

development and progression is not fully understood and conflicting results have been reported,[14–17] we decided to investigate the distribution of NGFR in breast tissue from various benign and malignant conditions and explore potential prognostic relationships. In a preliminary analysis, we and others[5] observed that NGFR showed a peculiar distribution, being restricted to myoepithelial cells, intralobular fibroblasts of terminal duct-lobular units, perivascular cells (vascular adventitia) and nerve bundles.

In several contexts of diagnostic breast pathology, the identification of myoepithelial cells is pivotal for an accurate diagnosis, namely to differentiate preinvasive from invasive breast lesions. For example, distinguishing ductal carcinoma in situ (DCIS) vs invasive ductal carcinoma, radial scar vs infiltrating tubular carcinoma, cancerisation of sclerosing adenosis by DCIS mimicking invasive breast carcinoma, adenoid cystic carcinoma vs collagenous spherulosis or cribriform DCIS, papillary carcinoma vs papilloma, and nipple adenoma vs invasive ductal carcinoma.[18–21] In recent years, several myoepithelial markers have been described, most of them either related to the basal nature of myoepithelial cells or to the smooth muscle apparatus of these cells.[18–22] However, the development of novel myoepithelial markers is not only important for diagnostic purposes but also for prognostication. There are several lines of evidence to suggest that high-grade breast carcinomas expressing basal/myoepithelial markers have distinct pathological features, expression profiles and, most importantly, clinical behaviour when compared to high-grade breast carcinomas devoid of basal-like differentiation.[23–30] The landmark studies of Perou et al[23] and Sorlie et al[24,25] revealed basal-like breast carcinomas to be more aggressive than oestrogen receptor (ER)-positive cancers, the former having significantly shorter disease-free and overall survival. Furthermore, recent data from our laboratory have suggested that basal tumours are heterogenous in terms of their molecular genetic profiles and that there might be a subgroup with a more favourable prognosis.[31]

We hypothesised that, as NGFR is consistently expressed by myoepithelial cells of the breast,[5] it would also be expressed by a subset of basal-like breast carcinomas. Furthermore, given that NGFR may block tumourigenesis and induce apoptosis in some systems, it seemed possible that basal-like breast carcinomas with NGFR expression would have a more favourable prognosis compared to NGFR-negative basal-like breast carcinomas.

Here we analysed a large series of benign and preinvasive breast lesions, biphasic tumours, tumours with myoepithelial differentiation and two cohorts of patients with breast cancer: a cohort of 245 breast cancer patients treated with anthracycline-based adjuvant chemotherapy and 37 basal-like breast carcinomas as defined by the immunohistochemical panel proposed by Nielsen et al.[26]

Materials and methods

Cases

Samples of normal breast tissue and benign and malignant breast lesions were retrieved from the archives of the Royal Marsden Hospital, London, UK, with appropriate local Ethical Committee approval. All cases were reviewed by experienced pathologists (JSRF, SDP) and graded according to a modified version of Scarff-Bloom–Richardson system.[32]

Normal breast and benign breast lesions
Representative tissue sections of benign and pre-invasive breast lesion series comprised 10 normal breast tissue samples obtained from mammoplasties, 11 papillomas, 12 radial scars, 13 sclerosing adenosis and five pseudoangiomatous stromal hyperplasias.

Biphasic neoplasms
Representative sections of 18 fibroadenomas, eight benign phyllodes tumours and three malignant phyllodes tumours were retrieved from the files of the Royal Marsden Hospital.

Tumours with myoepithelial differentiation
Representative sections of two adenomyoepitheliomas and 20 metaplastic breast carcinomas (reviewed by JSRF, FM and FCS and reclassified as seven spindle cell carcinomas,[33] two carcinomas with heterologous elements,[34] six carcinomas with squamous metaplasia[35] and five matrix-producing breast carcinomas[36]) were analysed.

Breast cancer precursors and preinvasive lesions
Putative breast cancer precursors and preinvasive lesions included nine hyperplasias of usual type, 14 columnar cell lesions/flat atypia, 40 DCIS and 10 lobular carcinomas in situ (nine classic lobular carcinomas in situ and one pleomorphic lobular carcinoma in situ).

Invasive breast carcinomas
Apart from malignant tumours with myoepithelial differentiation, two cohorts of invasive carcinomas were analysed:

(i) *Invasive breast carcinomas of usual type*: The first cohort was included in duplicate in two tissue microarray blocks and comprised 245 invasive breast carcinomas (185 invasive ductal carcinomas, 27 invasive lobular carcinomas, 25 invasive mixed carcinomas and eight invasive breast carcinomas of other special types). Two tissue microarray blocks containing 245 invasive breast carcinomas from 245 patients were constructed in duplicate as previously described.[18] In brief, 0.6 mm core tissue specimens were taken from selected areas of donor blocks (ie, original tumour blocks) and precisely

arrayed into two new recipient paraffin blocks ($20 \times 35 \, mm^2$) with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD, USA). The presence of tumour tissue in the arrayed sample was verified on a haematoxylin- and eosin-stained (H&E) section. In the tissue microarray cohort, NGFR expression was correlated with various clinicopathological parameters, including age at diagnosis, tumour size, tumour grade, presence of vascular invasion, presence of lymph node metastasis, local recurrence, distant metastasis, disease-free and overall survival. NGFR expression was also correlated with that of several immunohistochemical markers, including ER, progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2) and epidermal growth factor receptor (EGFR). Follow-up was available for 244 patients, ranging from 0.5 to 135 months (median = 67 months, mean = 67 months).

(ii) *Basal-like breast carcinomas*: The second cohort was analysed on representative whole tissue sections and comprised 37 breast carcinomas with basal-like phenotype. In this study, basal-like phenotype was defined as described Nielsen et al.[26] Briefly, all cases were grade 3 invasive ductal carcinomas lacking HER2 and ER and showing positivity for EGFR and/or cytokeratin 5/6.[26] All patients were treated with initial surgery followed by anthracycline-based adjuvant chemotherapy. Follow-up was available for all patients, ranging from 8 to 133 months, with a mean of 63 months and median of 61 months.

Immunohistochemistry

Immunohistochemistry was performed on representative formalin-fixed, paraffin-embedded tissue sections which were stained for NGFR with the antibody NGFR5 (Abcam ab-3125, Cambridge, UK).[5] Sections were dewaxed in xylene, taken through ethanol (99.7–100% v/v) and subjected to high-temperature antigen retrieval (18 min of microwaving in citrate buffer (pH 6.0)). Slides were allowed to cool for 20 min at room temperature and then incubated in Envision + peroxidase blocking solution (Dakocytomation, Glostrup, Denmark) for 5 min and rinsed with 0.05% Tris-buffered saline (TBS)/ Tween 20 buffer pH 7.4 and primary antibody was applied (1:200) for 30 min at room temperature. The primary antibody was rinsed off in 0.05% Tween 20 in TBS (pH 7.4). Detection was achieved with the DAKO Envision + /HRP system (Dakocytomation). Positive controls (nerve bundles of skin, melanoma) and negative controls (omission of the primary antibody and IgG-matched serum) were performed for each immunohistochemical run.

The distribution of NGFR in tissue sections was assessed by three of the authors (JSRF, DS and SDP) on a multihead microscope. A consensus score was assigned for each case. For the benign and pre-invasive lesions, the distribution and intensity of NGFR staining in the luminal/epithelial and myo-epithelial cell compartments were evaluated semi-quantitatively as previously described (distribution: 1 = <5% of cells stained, 2 = 5–25%, 3 = 25–50% and 4 ≥ 50%; intensity: 0 = negative, + = weak, + + = moderate and + + + = strong).[18] Invasive carcinomas displaying moderate-to-strong membrane staining with or without cytoplasmic reactivity in bona fide tumour cells were considered positive. The distribution pattern of NGFR in stromal cells of invasive carcinomas was also recorded. Only membranous with or without cytoplasmic staining was considered specific.

To compare the distribution of NGFR with the expression of other myoepithelial and stromal markers in normal breast and selected benign breast lesions, 10 samples were subjected to immunohistochemistry (as described above) with antibodies for p63 (4A4, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-smooth muscle actin (ASMA, 1A4, 1:200, Dako, Glostrup, Denmark), calponin (CALP, 1:25, Novocastra, Biogenex, San Ramon, CA, USA), Ck14 (LL02, 1:40, Novocastra, Newcastle-upon-Tyne, UK), Ck5/6 (D516B4, 1:600, Chemicon, Temecula, CA, USA), smooth muscle myosin heavy chain (SMM-HC, SMMS-1, 1:100, Dakocytomation), CD34 (QBEend10, 1:30, Dakocytomation) and vimentin (V9, 1:500, Dakocytomation).

To further corroborate the distribution of NGFR in myoepithelial cells, double immunolabelling with NGFR and p63 was carried out in four samples of normal breast and three samples of DCIS using the Envision double labelling system (Dakocytomation) according to the manufacturer's instructions. p63 was chosen because it is consistently expressed in nuclear compartment of normal myoepithelial cells,[19,37] allowing an objective assessment of NGFR (membrane) and p63 (nucleus) coexpression. Briefly, slides were subjected to high-temperature antigen retrieval (18 min of microwaving in citrate buffer (pH 6.0)), allowed to cool for 20 min at room temperature and then incubated in Envision + peroxidase blocking solution (Dakocytomation) for 5 min, rinsed in water and transferred to TBS. Sections were incubated for 30 min with the first primary antibody NGFR (1:200) and rinsed in TBS. A secondary linking polymer antibody bound to horseradish peroxidase was then applied for 30 min. Sections were rinsed with TBS and developed with liquid DAB plus (Dakocytomation) for 10 min (brown). After completion of the primary staining sequence, slides were rinsed in TBS and a double stain block solution was applied for 3 min (Dakocytomation), followed by rinsing in TBS. p63 was applied (1:25) to the tissue sections for 30 min in an incubation chamber and slides were again rinsed in TBS. A secondary linking polymer antibody bound to alkaline phosphatase was added for 30 min,

followed by rinse in TBS. Sections were developed with a substrate-fast red chromogen solution (red), rinsed in TBS, counterstained in Mayer's haematoxylin for 10 s and mounted in aqueous mounting medium.

The expression of basal/myoepithelial markers Ck5/6, Ck14, Ck17 (E3, 1:100, Dakocytomation); EGFR (31G7, 1:50; Zymed); ER (1D5, 1:40, Dakocytomation), PR (PGR636, 1:150, Dakocytomation) and HER2 (Herceptest, Dakocytomation) was analysed in the two cohorts of patients. For tissue microarray and whole tissue section immunohistochemical analysis, a cutoff of 10% unequivocally stained neoplastic cells was adopted for ER, PR, basal markers and EGFR,[30] whereas HER2 was scored according to the Herceptest scoring system.[38] For basal markers, only cytoplasmic staining was considered specific, for EGFR and HER2 only membranous staining was regarded as specific and for ER and PR, only nuclear staining was considered specific.

Statistical Analysis

The Statview software package was used for all calculations. Correlations between categorical variables were performed using the χ^2 test and Fisher's exact test. Correlations between continuous and categorical variables were performed with analysis of variance (ANOVA). Disease-free and overall survival was expressed as the number of months from diagnosis to the occurrence of an event (local recurrence/metastasis and disease-related death, respectively). Cumulative survival probabilities were calculated using the Kaplan–Meier method. Differences between survival rates were tested with the log-rank test. All tests were two-tailed, with a confidence interval of 95%.

Multivariate analysis was performed using the Cox multiple hazards model. A P-value of 0.05 in the univariate survival analysis was adopted as the limit for inclusion in the multivariate model. Cases with missing values were excluded in the multivariate analysis model.

Results

Normal and Benign Breast Lesions

(i) Normal breast tissue and fibrocystic changes
NGFR was expressed consistently in myoepithelial cells arranged as a continuous layer around ducts and lobular units, whereas luminal epithelial cells were devoid of any staining (Figure 1). The distribution of NGFR in normal myoepithelial cells was similar to that of traditional myoepithelial markers (p63, α-smooth muscle actin, smooth
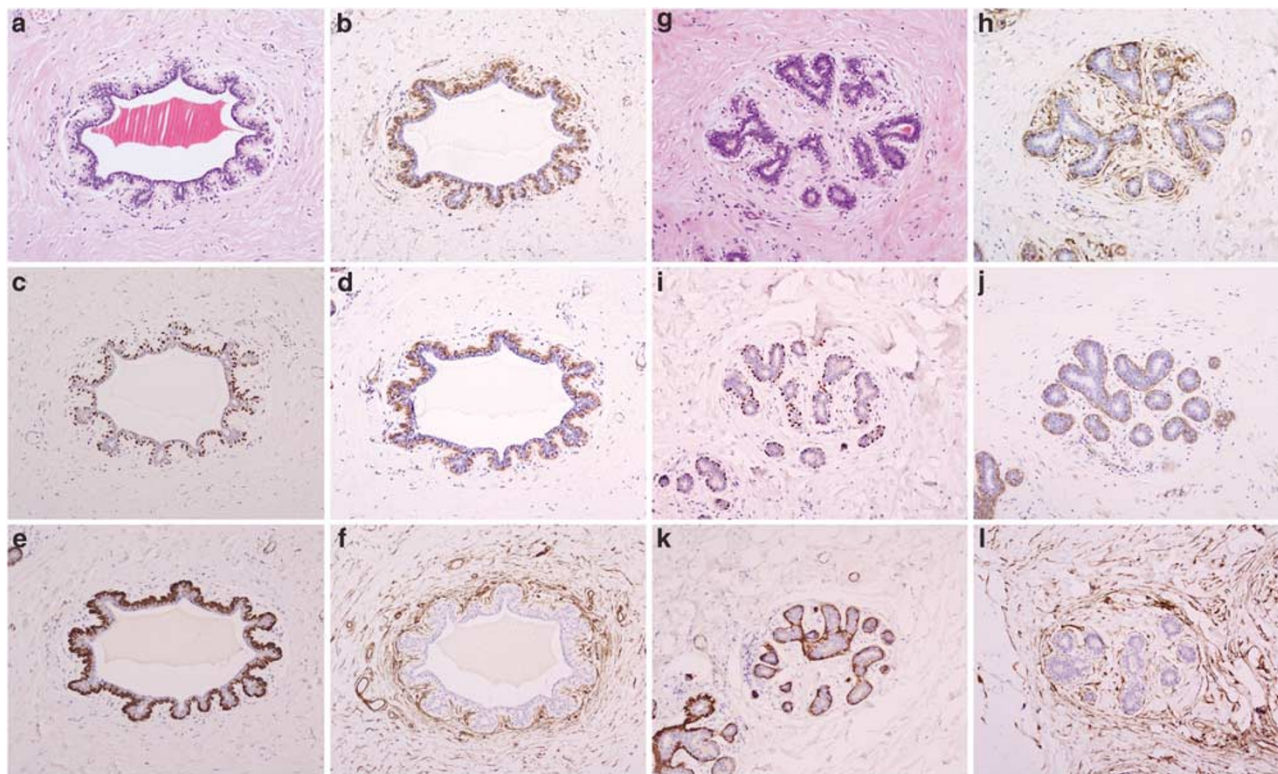

Figure 1 Serial sections of normal breast duct (**a–f**) and acini (**g–l**). NGFR (**b** and **h**), p63 (**c** and **i**), α-smooth muscle actin (**d** and **j**) and SMM-HC (**e** and **k**) displayed strong and consistent positivity in myoepithelial cells. Note that NGFR (**h**) also stained perivascular cells and CD34-positive (**f** and **l**) intralobular fibroblasts. (**a** and **g**, H&E, **b–f** and **h–l**, Envision + /DAB).

muscle myosin heavy chain). Ductal myoepithelial cells were consistently strongly decorated by NGFR, whereas myoepithelial cells of the lobules occasionally showed moderate-to-strong staining. Double immunostaining with antibodies for NGFR and p63 revealed a colocalisation of these two markers in myoepithelial cells.

The stromal compartments of the breast showed a differential distribution of NGFR: intralobular fibroblasts (ie, fibroblasts of the modified stroma) showed strong membranous staining for NGFR, whereas interlobular fibroblasts and periductal fibroblasts were either negative or showed weak and focal staining. Nerve bundles were uniformly positive for NGFR. Perivascular cells (vascular adventitia) of medium-sized and large vessels also showed membranous positivity for NGFR, whereas perivascular cells of small vessels and endothelial cells of capillaries were either negative or were weakly decorated by this marker (Figure 1).

Given that nerve bundles and perivascular cells (vascular adventitia) of medium-sized and large vessels were consistently positive for NGFR, immunohistochemical staining for NGFR in these structures served as reference staining and internal control for the experiments.

Apocrine cysts and apocrine metaplasia ($n = 8$) showed a distribution of NGFR similar to that of normal breast (ie, a continuous layer of NGFR + myoepithelial cells surrounding NGFR-apocrine cells), however, the intensity varied from moderate-to-high. This subtle decrease of NGFR staining was also observed in dilated ducts of specimens with fibrocystic change.

(ii) Benign breast lesions
Table 1 summarises the distribution of NGFR in intraductal papillomas, radial scars, sclerosing adenosis, fibroadenomas, benign phyllodes tumours and adenomyoepitheliomas.

(a) *Intraductal papilloma*: NGFR was strongly positive in the myoepithelial cells located between the luminal epithelial compartment and the fibrovascular cores of the papillae (Figure 2a–c) and in the outer layer of tubules entrapped in the fibrovascular stroma. The luminal compartment of nine cases completely lacked NGFR staining, whereas two cases harboured <5% of NGFR-positive luminal cells. Small vessels in the papillary cores were negative for NGFR, whereas medium-sized vessels in sclerotic areas showed perivascular positivity, similar to that observed in normal breast samples.

(b) *Radial scar*: NGFR was positive in the outer myoepithelial compartment of all cases, and notably in the ducts entrapped in the central elastotic areas. Luminal epithelial cells consistently lacked NGFR staining. Although NGFR showed focal positivity in stromal myofibroblasts located at the central aspects of radial scars of two cases, the staining was not as strong as that observed with α-smooth muscle actin (Figure 2–f). Scattered medium-sized and large vessels showed perivascular positivity for NGFR.

(c) *Sclerosing adenosis*: The myoepithelial compartment of sclerosing adenosis was consistently strongly and diffusely positive for NGFR. The staining was even more conspicuous in the areas with myoid metaplasia. Focal NGFR staining in the luminal compartment was observed in 1 case (Figure 2g–i). Myofibroblasts showed weak-to-moderate staining in five cases, however, in none of them the staining was as strong as that observed in myoepithelial cells.

(d) *Pseudoangiomatous stromal hyperplasia*: Five samples of pseudoangiomatous stromal hyperplasias were studied. In all samples, myofibroblasts showed strong and diffuse immunoreactivity for NGFR, with a distribution similar to that described for CD34[39] (Figure 3a and b).

Biphasic Neoplasms

NGFR showed strong positivity in the myoepithelial cell compartment of all fibroadenomas and benign phyllodes tumours, whereas luminal cells were devoid of any staining in all cases. NGFR showed a peculiar distribution in the stromal compartment of 16/18 fibroadenomas and 6/8 benign phyllodes tumours: stromal cells arranged in concentric layers

Table 1 NGFR distribution in benign breast lesions

| Lesion | | Myoepithelial compartment | | | | | | | | | Luminal compartment | | | | | | | | |
| | | NGFR (intensity) | | | | NGFR (distribution) | | | | | NGFR (intensity) | | | | NGFR (distribution) | | | | |
	n	0	1+	2+	3+	0	1+	2+	3+	4+	0	1+	2+	3+	0	1+	2+	3+	4+
Papilloma	11	0	0	0	11	0	0	0	1	10	9	1	1	0	9	2	0	0	0
Radial scar	12	0	0	0	12	0	0	0	0	12	11	0	1	0	11	1	0	0	0
Sclerosing adenosis	13	0	0	0	13	0	0	0	0	13	12	1	0	0	12	1	0	0	0
Fibroadenoma	18	0	0	0	18	0	0	0	0	18	13	3	2	0	13	5	0	0	0
Benign phyllodes tumour	8	0	0	0	8	0	0	0	0	8	8	0	0	0	8	0	0	0	0
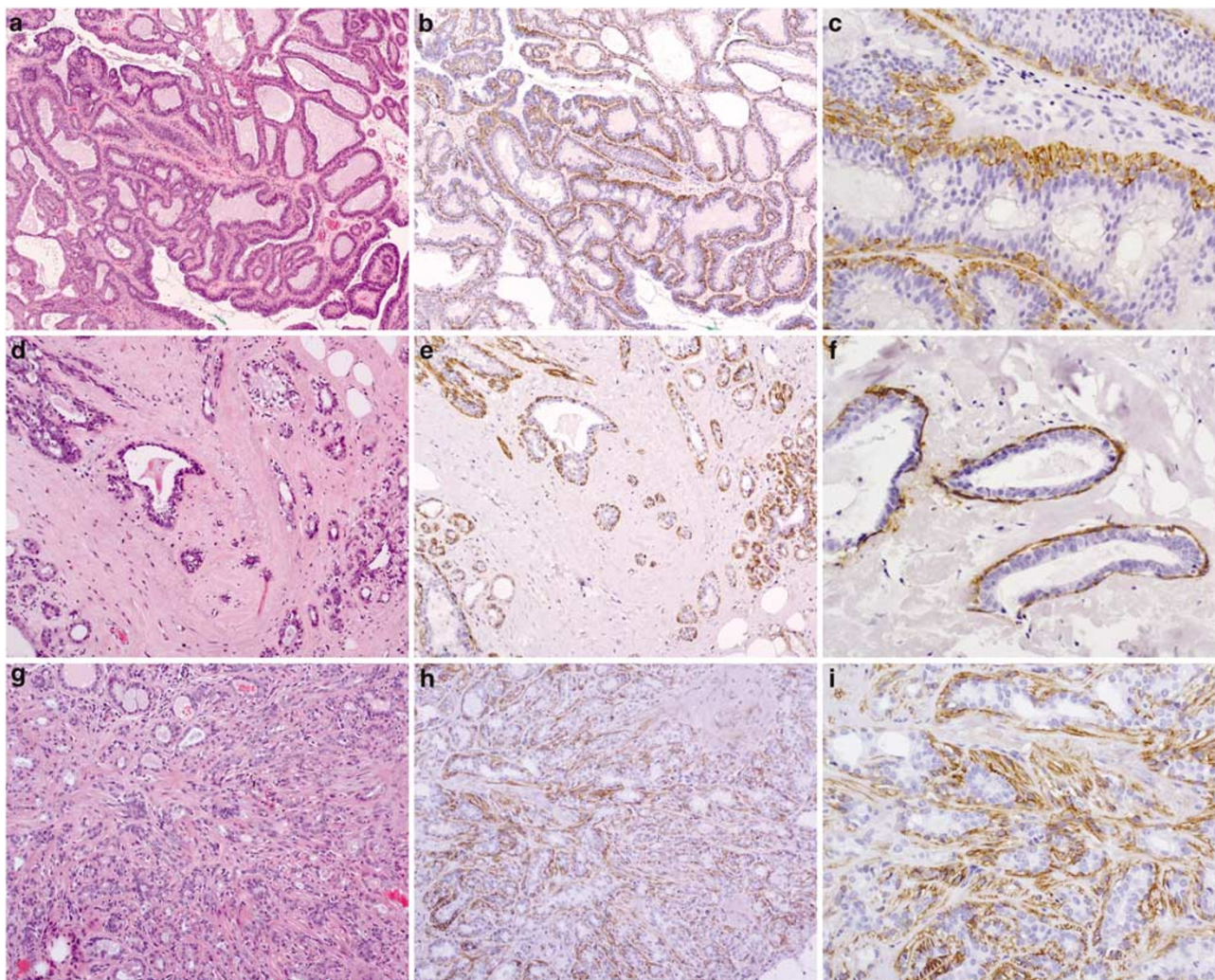
Figure 2 NGFR expression in benign breast lesions. Low-power magnification of a papilloma (**a**, H&E) showing NGFR expression in myoepithelial cells (**b**, Envision + /DAB). Note the specific staining in the continuous layer of myoepithelial cells and lack of staining in luminal and stromal cells (**c**, Envision + /DAB). Medium-power magnification of the central area of a radial scar (**d**, H&E). Note the reactivity of NGFR in myoepithelial cells surrounding ducts in sclerotic zones (**e** and **f**, Envision + /DAB). Medium-power magnification of a sclerosing adenosis (**g**, H&E). NGFR decorated the spindle-shaped and epithelioid myoepithelial cells (**h** and **i**, Envision + /DAB). Scattered stromal cells displayed weak NGFR staining (**h**, Envision + /DAB).

around ductal structures showed a stronger staining when compared to other areas of the stromal component (Figure 3c and d). In the remaining cases, which harboured a more collagenous stromal compartment, NGFR was restricted to perivascular cells.

Tumours with Myoepithelial Differentiation

In two adenomyoepitheliomas, the outer clear and spindle-shaped myoepithelial cells displayed strong reactivity for NGFR. In contrast, the epithelial cells of these two cases showed rather weak or focal expression.

Metaplastic breast carcinomas, tumours known to harbour features of myoepithelial differentiation,[33,36,37,40–44] were positive for NGFR in nine out

of 20 cases (45%). These included four out of five positive matrix producing carcinomas (Figure 5a and b), one out seven spindle cell carcinomas (Figure 5c and d), three out of six carcinomas with squamous metaplasia and one out of two carcinoma with heterologous elements.

Preinvasive Lesions (Ductal Hyperplasia of Usual Type, Columnar Cell Lesions/Flat Atypia, Lobular Carcinoma *In Situ* and DCIS)

Table 2 summarises the distribution of NGFR in putative breast cancer precursors and preinvasive lesions. All hyperplasias of usual type exhibited a continuous or near-continuous layer of strongly NGFR-stained myoepithelial cells. The solid areas of proliferating hyperplastic cells were focally, weakly-to-moderately positive for NGFR in six out
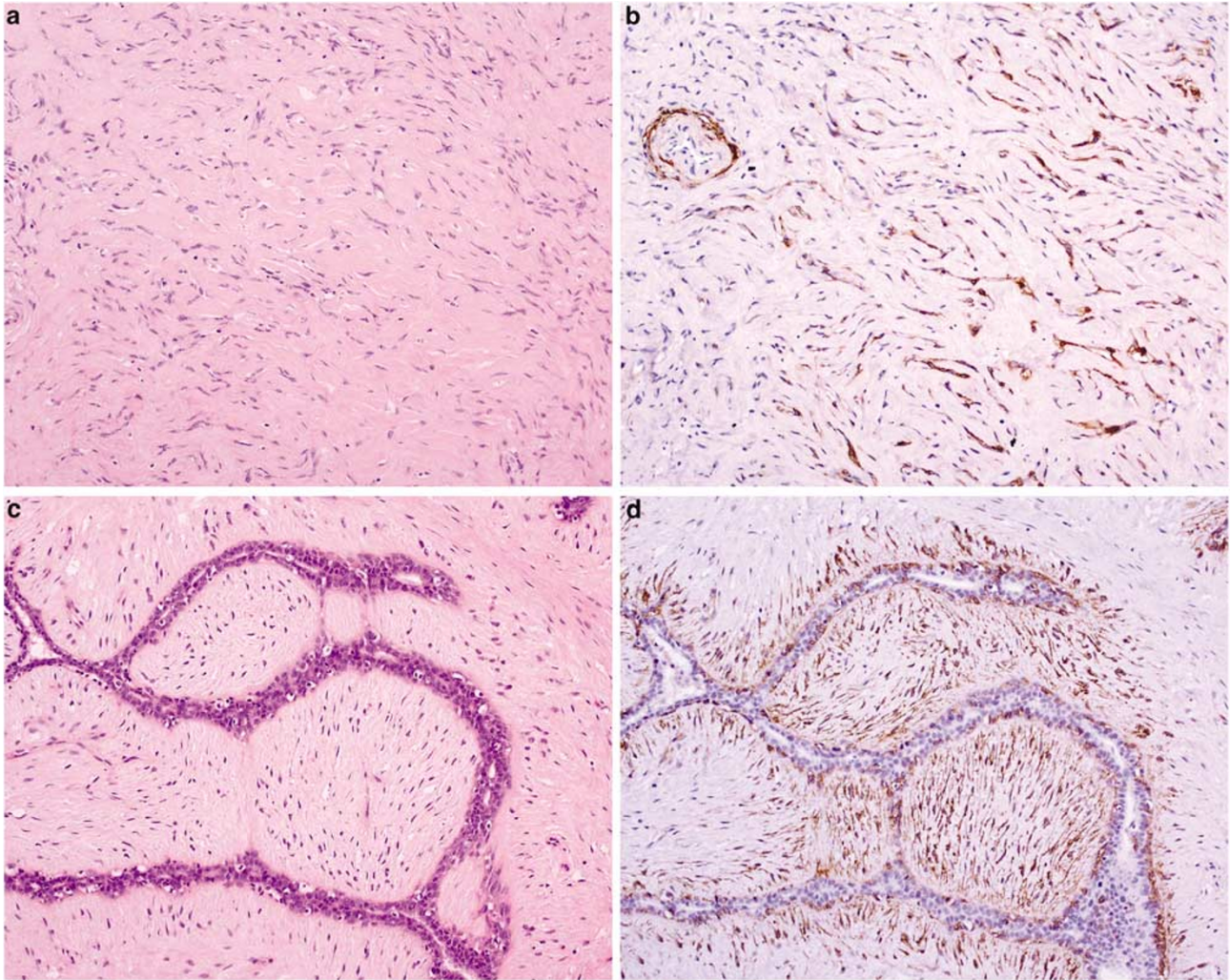
Figure 3 NGFR distribution in pseudoangiomatous stromal hyperplasia (**a**, H&E; **b**, Envision + /DAB) and fibroadenoma (**c**, H&E; **d**, Envision + /DAB).

Table 2 NGFR distribution in myoepithelial and luminal cells of putative precursors and preinvasive lesions

Lesion	Nuclear grade	Type	n	NGFR—myoepithelial cells Intensity				Distribution					NGFR—luminal cells Intensity				Distribution				
				0	1+	2+	3+	0	1+	2+	3+	4+	0	1+	2+	3+	0	1+	2+	3+	4+
HUT	—	—	9	0	0	1	8	0	0	0	1	8	3	4	2	0	3	5	1	0	0
CCL	—	—	14	0	0	3	11	14	0	0	0	14	0	0	0	0	14	0	0	0	0
LCIS	1	—	9	0	0	3	6	0	0	0	3	6	9	0	0	0	9	0	0	0	0
PLCIS	3	—	1	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0
DCIS	1	Cribriform	4	0	0	1	3	0	0	0	0	4	4	0	0	0	4	0	0	0	0
DCIS	2	Cribriform	2	0	0	1	1	0	0	0	0	2	2	0	0	0	2	0	0	0	0
DCIS	2	Cribriform/solid	3	0	0	2	1	0	0	0	1	2	3	0	0	0	3	0	0	0	0
DCIS	2	Micropapillary	2	0	0	1	1	0	0	0	0	2	2	0	0	0	2	0	0	0	0
DCIS	2	Solid	2	0	0	0	2	0	0	0	0	2	2	0	0	0	2	0	0	0	0
DCIS	2	Solid/comedo	3	0	0	0	3	0	0	0	0	3	3	0	0	0	3	0	0	0	0
DCIS	3	Comedo	13	0	0	2	11	0	0	0	0	13	12	0	0	1	12	1	0	0	0
DCIS	3	Cribriform/micropapillary/solid	1	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0
DCIS	3	Cribriform/comedo	1	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0
DCIS	3	Micropapillary	1	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0
DCIS	3	Papillary	2	0	0	0	2	0	0	0	0	2	2	0	0	0	2	0	0	0	0
DCIS	3	Solid	6	0	0	1	5	0	0	0	1	5	6	0	0	0	6	0	0	0	0

CCL: columnar cell change/flat atypia; DCIS: ductal carcinoma *in situ*; HUT: hyperplasia of usual type; LCIS: lobular carcinoma *in situ*.
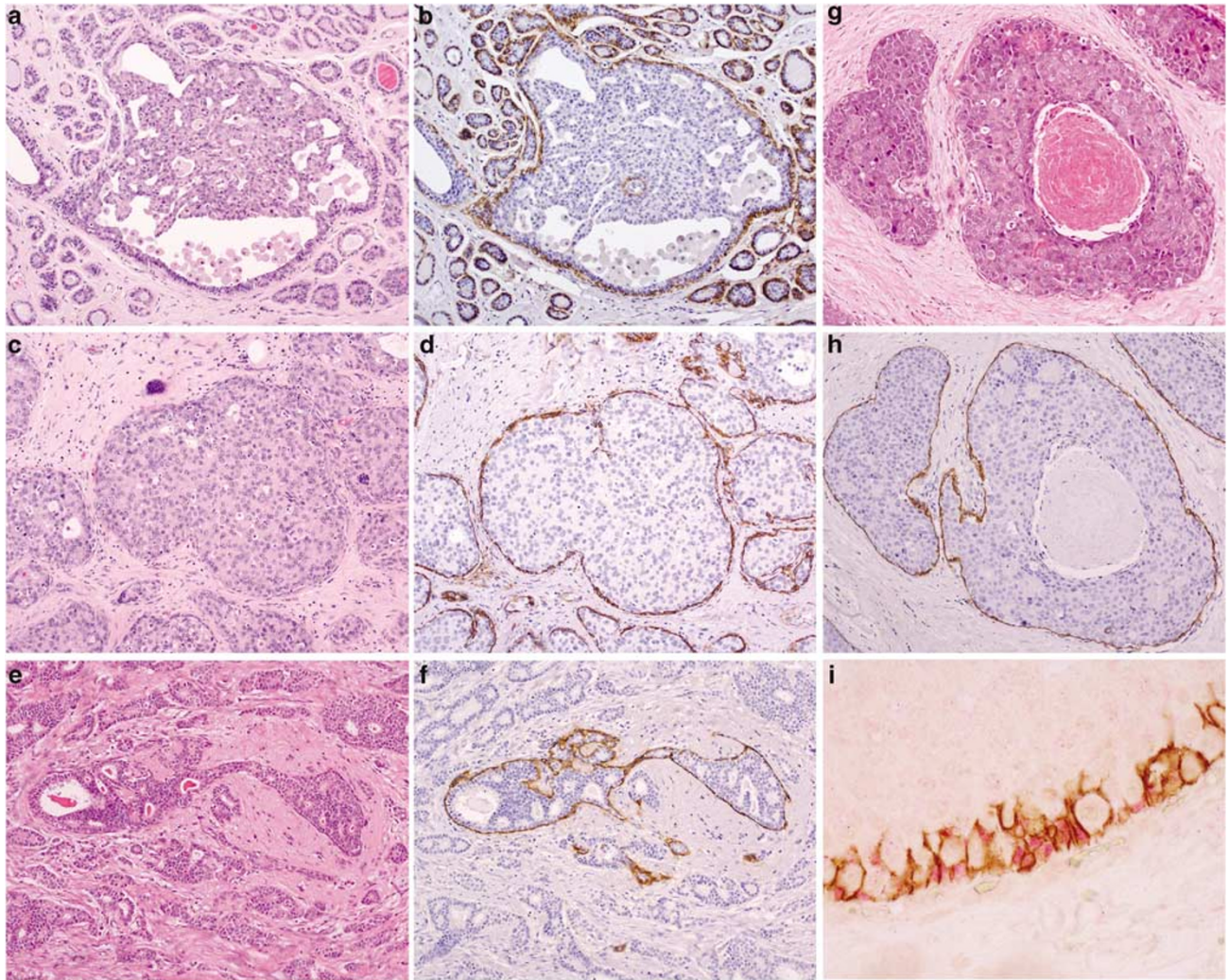
Figure 4 NGFR expression in putative breast cancer precursors and preinvasive breast lesions. Hyperplasia of usual type. Note the presence of NGFR-positive myoepithelial cells surrounding ductal structures and admixed with the hyperplastic population (**a**, H&E; **b**, Envision + /DAB). Grade 2, cribriform and solid ductal carcinoma *in situ* (DCIS) (**c**, H&E; **d**, Envision + /DAB). Note the presence of a continuous layer of myoepithelial cells surrounding the ducts. Low grade, cribriform DCIS entrapped in an invasive cribriform/tubular carcinoma of the breast. Observe the presence of a continuous layer of myoepithelial cells and scattered residual myoepithelial cells partially surrounding invasive ducts (**e**, H&E; **f**, Envision + /DAB). Grade 3, comedo DCIS (**g**, H&E; **h**, Envision + /DAB). Double immunostaining demonstrating coexpression of p63 (red) and NGFR (brown) in myoepithelial cells of a grade 3, comedo DCIS (**i**, Envision double staining system/no counterstaining).

of nine cases (Figure 4a and b). Conversely, the neoplastic luminal compartment of columnar cell lesions/flat atypia, lobular carcinomas *in situ* and DCIS was devoid of any staining in all but one case of high-grade DCIS. In these lesions, NGFR immunoreactivity was largely restricted to myoepithelial cells surrounding ducts and lobular units (Figure 4c–h). Scattered NGFR-positive periductal fibroblasts were observed in four hyperplasias of usual type, three columnar cell lesions/flat atypia, four lobular carcinomas *in situ* and eight DCIS, but these cells neither formed a continuous layer surrounding the ductal structures nor showed staining of comparable intensity with that of myoepithelial cells. In four lobular carcinomas *in situ*, NGFR-positive cytoplasmic projections of myoepithelial cells

encasing individual lobular carcinomas *in situ* cells were observed, which at first glance mimicked neoplastic cell positivity. However, on closer inspection, cell membranes of neoplastic cells were devoid of NGFR staining. Double immunostaining with antibodies for NGFR and p63 demonstrated coexpression of these two markers in the continuous layer of myoepithelial cells surrounding neoplastic cells of the three high-grade DCIS analysed.

Invasive Breast Carcinomas

(I) Invasive carcinomas of usual type
Tissue microarray: In 22 of the 245 cases, cores were either missing on the tissue microarray or NGFR was

not interpretable on the duplicate tissue cores. In all, 11 out of the remaining 223 cases (4.9%) showed NGFR immunoreactivity. These included nine bonafide cases of grade 3 invasive ductal carcinomas and two invasive ductal carcinomas with minor components of metaplastic elements. The prevalence of NGFR positivity was significantly higher in invasive ductal and metaplastic carcinomas than in lobular and mixed carcinomas ($P < 0.0001$). In this cohort of patients, NGFR showed statistically significant correlations with Ck14 ($P < 0.0001$), Ck5/6 ($P < 0.005$), Ck17 ($P < 0.0001$) and EGFR ($P < 0.0001$) and inverse associations with ER ($P < 0.0001$) and PR ($P < 0.0001$) (Table 3). In fact, all NGFR-positive cases were ER negative, high-grade invasive breast carcinomas.

NGFR expression showed a direct association with grade ($P < 0.05$), an inverse association with the presence of lymph node metastasis at diagnosis ($P < 0.005$) and a borderline inverse correlation with the presence of lymphovascular invasion ($P = 0.0525$) (Table 3). Tumour size and age at diagnosis displayed no correlation with NGFR expression.

Survival analysis revealed positivity for basal markers, presence of lymph node metastasis, grade 3, ER- and PR-positive tumours to be associated with shorter disease-free and overall survival in univariate analysis (data not shown). In multivariate analysis, grade 3, lack of lymph node metastasis and negativity for basal markers were independent prognostic factors for disease-free survival, whereas presence of lymph node metastasis and positivity for basal markers were independent prognostic factors for overall survival (data not shown). NGFR showed no association with disease-free or overall survival in the whole cohort (data not shown).

Stromal cells (myofibroblasts) in the central aspects of all but two invasive ductal carcinomas were devoid of NGFR staining, however, perivascular staining was observed in all cases. In tissue adjacent to invasive tumours, scattered positive myofibroblasts could be observed in the majority of samples. In four lobular carcinomas, scattered myofibroblasts admixed with tumour cells displayed strong membrane staining. This pattern was observed where remains of terminal duct-lobular units were entrapped and partially destroyed by neoplastic cells. In invasive breast carcinomas, no association between stromal cell positivity and any clinicopathological parameter was identified (data not shown).

(II) Basal-like breast carcinomas
As NGFR positivity was restricted to ER negative (11/11 cases) and 10 of these cases showed expression of basal-like immunohistochemical markers, the prognostic impact of NGFR was assessed in a second cohort of 37 high-grade ductal carcinomas classified as basal-like according to Nielsen et al[26]

Table 3 Correlation of NGFR with other parameters and immunohistochemical markers in invasive breast carcinomas analysed by tissue microarrays

Parameter	n	NA	NGFR+	NGFR−	Statistical association
Type					
IDC	222	23	9	158	$P < 0.0001$[a]
ILC			0	26	
Mixed			0	22	
Other			2	5	
Grade					
1	222	23	0	21	$P < 0.05$[a]
2			0	64	
3			11	126	
LVI					
+	221	24	4	140	$P = 0.0525$[b]
−			7	70	
LN mets					
+	215	30	2	133	$P < 0.005$[b]
−			9	71	
ER					
+	221	24	0	177	$P < 0.0001$[b]
−			11	33	
PR					
+	221	24	1	161	$P < 0.0001$[b]
−			10	49	
HER2					
+	220	25	0	173	$P > 0.05$[b]
−			11	36	
EGFR					
+	219	26	7	14	$P < 0.0001$[b]
−			4	194	
Ck 14					
+	221	24	6	14	$P < 0.0001$[b]
−			5	196	
Ck 5/6					
+	213	32	4	19	$P < 0.05$[b]
−			6	184	
Ck 17					
+	220	25	6	19	$P < 0.0001$[b]
−			5	190	
Basal markers					
−	223	22	10	23	$P < 0.0001$[b]
+			1	189	

Ck: cytokeratin; ER: oestrogen receptor; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; LN mets: lymph node metastasis at diagnosis; LVI: lympho-vascular invasion at diagnosis; Mixed: ductal mixed carcinoma; *n*: number of samples; NA: not assessable; PR: progesterone receptor.
[a]χ^2 test.
[b]Fisher's exact test.

immunohistochemical panel. In this cohort, NGFR expression was observed in 38% of the cases (Figure 5e–h) and was associated with lack of lymph node metastasis at diagnosis ($P < 0.001$).
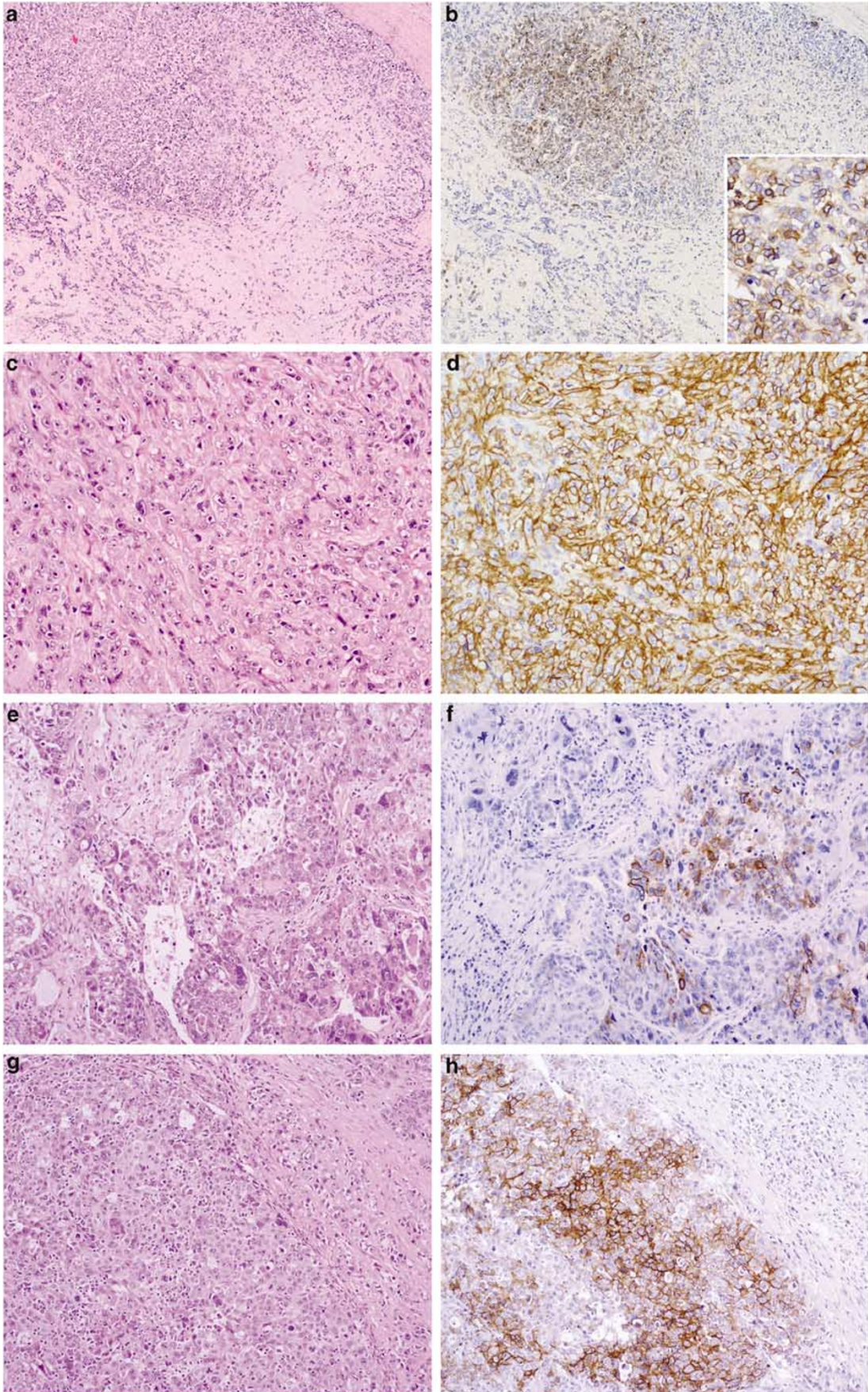
Discussion

In the present study, NGFR displayed remarkable sensitivity for myoepithelial cells of normal breast tissue as well as benign and malignant breast lesions, which was similar to that of traditional myoepithelial markers, such as α-smooth muscle actin, p63 and calponin. NGFR was not entirely specific, as it also stains fibroblasts/myofibroblasts of the specialised breast stroma, perivascular cells and neural bundles. However, NGFR expression in myofibroblasts was never as strong as that seen in myoepithelial cells and not as diffuse as that observed with α-smooth muscle actin.[19,21]

In the stromal compartment of normal breast and benign and malignant breast lesions, the distribution of NGFR is strikingly similar to that described for CD34.[39,45,46] In fact, NGFR was consistently expressed by intralobular (myo)fibroblasts, stromal cells of benign biphasic neoplasms and in pseudo-angiomatous stromal hyperplasias, but largely negative in the stromal compartment of invasive tumours.[39,45] Our findings corroborate those of Chauhan et al,[39] giving further evidence for the existence of more than one type of fibroblast in normal breast and in the stromal compartment of breast lesions.

In biphasic tumours of the breast, NGFR showed a peculiar distribution, with strong staining in the periductal/pericanalicular areas observed in 84.6% of benign lesions and absence of staining in three malignant phyllodes tumours. Similar expression patterns have been observed with other stromal markers, such as CD34.[39,47] Interestingly, the expression pattern of these markers has been linked with the clinical behaviour of these lesions.[47,48] Further studies analysing a large series of biphasic tumours of the breast are warranted.

NGFR was consistently positive in adenomyoepitheliomas and stained 45% of metaplastic breast carcinomas, tumours known to show myoepithelial differentiation.[33,36,37,40–44] NGFR positivity was significantly higher in metaplastic breast carcinomas when compared to that of a population-based cohort of breast cancers.

Most of the traditional myoepithelial markers related to the epithelial and basal characteristics of myoepithelial cells also stain tumours with squamous differentiation (eg, Ck5/6, 14 and p63 are frequently positive in squamous cell carcinomas).[19,37,49] On the other hand, some malignant tumours may harbour abortive myoepithelial differentiation, displaying poorly developed smooth muscle apparatus and frequently lacking expression of smooth muscle markers.[50] Therefore, based on our results, we advocate NGFR as an adjunct myoepithelial marker that in conjunction with other antibodies may be utilised to identify myoepithelial differentiation in breast neoplasms.

NGFR was positive in 11 out of 223 invasive breast carcinomas and all 11 were high grade, ER-negative breast carcinomas. Out of these cases, 10 showed expression of basal/myoepithelial markers (ie, Ck5/6, Ck14, Ck17 and EGFR).[19,21,26,27,30,31] Although NGFR showed no prognostic significance in this cohort, when its expression was analysed in a cohort of 37 basal-like breast carcinomas,[26] NGFR showed a statistically significant association with lack of lymph node metastasis. In addition, initial analysis in 37 basal-like breast carcinomas has revealed statistically significant associations between NGFR expression and longer disease-free and overall survival. These provisional results, although in a small number of patients, are promising and warrant further investigation of NGFR expression as a marker for basal-like breast carcinomas associated with a good prognosis.

Our results are in stark contrast with those of Descamps et al,[14] who demonstrated expression of NGFR in all breast carcinoma samples. However, in that study, NGFR expression was analysed at the mRNA level and tumours were neither microdissected nor analysed for the percentage of tumour cells in each sample.[14] Given that NGFR is consistently expressed in tumour stroma perivascular cells and occasionally in stromal cells, expression in all samples in that series would be reasonable, however, it would not reflect the expression of NGFR in neoplastic cells.

Jones et al[31] demonstrated that basal-like carcinomas as defined by cytokeratin 14 positivity may be subclassified into two prognostically significant categories. In that study basal-like tumours with a poor prognosis (cluster 1) showed a significantly higher frequency of 17q loss when compared to noncluster 1 (good prognosis) basal-like carcinomas. As NGFR gene maps to 17q21 and both lack of NGFR expression or deletion on 17q appear to be associated with a more aggressive behaviour in basal-like carcinomas, further studies directly addressing the correlation between NGFR expression and deletion of 17q21 are required to evaluate whether these deletions target NGFR or other putative tumour suppressor genes mapping to this region, namely BRCA1 or NM23.

In conclusion, we demonstrate that NGFR is preferentially expressed by myoepithelial and

Figure 5 NGFR expression in invasive carcinomas. Matrix-producing metaplastic breast carcinoma (a, H&E; b, Envision + /DAB). Inset: note the presence of membrane staining in neoplastic cells. Metaplastic spindle cell carcinoma (c, H&E; d, Envision + /DAB). Grade 3 oestrogen receptor (ER) negative basal-like invasive ductal carcinoma (e, H&E; f, Envision + /DAB) showing focal positivity for NGFR. Grade 3 oestrogen receptor negative basal-like invasive ductal carcinoma with pushing borders (g, H&E; h, Envision + /DAB) showing diffuse positivity for NGFR.

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intralobular fibroblasts of normal breast, benign breast lesions and preinvasive lesions. Owing to its high sensitivity and considerable specificity, we advocate the use of NGFR as an adjunct myoepithelial marker in panels designed for the identification of myoepithelial cells in benign, preinvasive lesions and tumours with myoepithelial differentiation. Initial analyis of NGFR expression in 37 basal-like breast carcinomas suggest that this may be a marker for basal-like breast carcinomas associated with a good prognosis. These findings give further indirect evidence to the reported tumour/metastasis suppressor properties of NGFR.[7–13] Furthermore, studies addressing the prognostic impact of NGFR expression in basal-like carcinomas are warranted.

Acknowledgements

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