

Expression patterns among interferon regulatory factor-1, human X-box binding protein-1, nuclear factor kappa B, nucleophosmin, estrogen receptor-alpha and progesterone receptor proteins in breast cancer tissue microarrays

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Abstract. Interferon regulatory factor-1 (IRF-1), human X-box binding protein-1 (hXBP-1), nuclear factor kappa B p65 (NFκB p65) and nucleophosmin (NPM) have been implicated in a signaling network of endocrine responsiveness. Expression of these proteins was measured by immunohistochemistry in tissue microarrays of 54 breast tumors. Correlations between each protein and established prognostic markers were assessed by Spearman's rank order correlation coefficient and partial correlation coefficient analyses. Moderate/strong staining is seen for hXBP-1 (79% of tumors) and NFκB p65 (57%). NPM exhibits nuclear staining (95%); IRF-1 exhibits both cytosolic (IRF-1c; 90%) and nuclear staining (IRF-1n; 51%). IRF-1c is associated with age ($p=0.034$); IRF-1n and PgR expression are correlated ($p=0.014$). NFκB p65 shows a borderline association with S phase ($p=0.062$). Coexpression of IRF-1c and hXBP1 ($p=0.001$), IRF-1c and NFκB ($p=0.002$), and hXBP-1 and NFκB ($p=0.018$) is observed. An inverse correlation exists between IRF-1n and NFκB ($p=0.034$). All four proteins are detected in breast tumors and their expression patterns support their role(s) in a key signaling network.

Introduction

Endocrine therapy, usually either the antiestrogen, Tamoxifen (TAM), ovariectomy or more recently an aromatase inhibitor or one of the newer selective estrogen receptor modulators

(SERM) or 'pure' antiestrogens, is an effective means to manage hormone-dependent breast cancer (1-3). An understanding of the mechanisms of resistance to endocrine therapies could identify better ways to predict responsiveness. We have previously hypothesized that endocrine responsiveness is affected by a complex gene network, rather than the activity of only one or two genes or signaling pathways (4-6). To identify the key components of such a network, we first derived variants of the MCF-7 human breast cancer cell line with different estrogen (7,8) and antiestrogen response profiles (9,10). Initial transcriptome and proteome analyses of these variants implicate several genes in endocrine resistance, including interferon regulatory factor-1 (IRF-1) (11,12), nuclear factor kappa B p65 (NFκB) (11,13), human X-Box binding protein-1 (hXBP-1) (11) and nucleophosmin (NPM) (14,15), which appear to function as part of a broader gene expression network (Fig. 1).

In the proposed network, NPM is predicted to inhibit IRF-1 activity, which reduces the ability of IRF-1 to activate apoptosis, most likely through inducing a caspase cascade. Inhibition of IRF-1 activity also may eventually contribute to increased activity of the survival factor NFκB (Fig 1). Increased NFκB may, in turn, induce a second survival factor, hXBP-1 (16). Evidence from experimental models has begun to show the likely functional relevance of the altered IRF-1 (12), NFκB (11,13,17), and hXBP-1 activities (Gomez BP, *et al*, Proc Am Assoc Cancer Res, abs. 3498, 2004) in affecting endocrine responsiveness. Studies to identify other members of this network and their interrelationships are currently in progress. The known functions of the key components of the network are described below.

IRF-1 is a transcription factor that exhibits tumor suppressor activities in several cancers (18,19). In breast cancer cells, IRF-1 signaling can reduce both the rate of cell proliferation and the incidence of human breast cancer xenografts in athymic nude mice (Bouker KB, *et al*, Carcinogenesis, In press). We have shown that a dominant negative IRF-1 blocks

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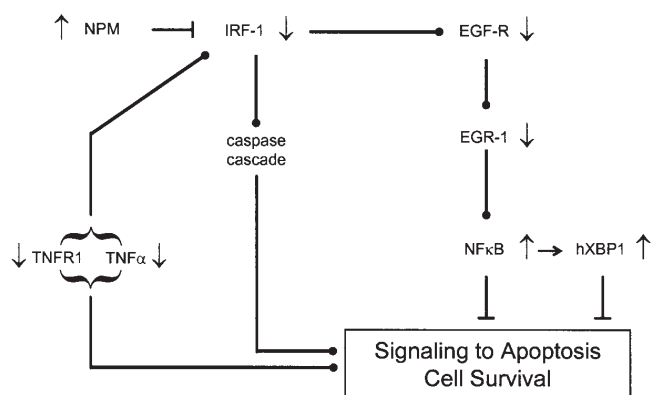


Figure 1. Components of a putative signaling network associated with endocrine responsiveness in breast cancer cells (adapted from ref. 6). ↑, increased; ↓, decreased; ⊥, blocks; —•, reduced ability to affect target. The network component incorporates the known protein/protein interactions between NPM and IRF-1 (NPM binds to IRF-1 and inhibits IRF-1 activity) and the predicted regulation of hXBP-1 by NFκB. Down-regulation of IRF-1 activity would reduce the activity of IRF-1/NFκB heterodimers that are known to regulate the transcription of several genes implicated in breast cancer, such as RANTES, VCAM-1, and IL-6. Down-regulation of TNFR1 (tumor necrosis factor receptor 1) and its ligand, TNFα (tumor necrosis factor alpha), were previously described (11), signaling from this complex is a major inducer of IRF-1 transcription. A full description of this network component and its anticipated function can be found in ref. 6.

antiestrogen-induced apoptosis in sensitive breast cancer cells and reduces their antiestrogen sensitivity (12); a similar role for IRF-1 has been recently reported in normal mammary cells (20). These activities of IRF-1 are probably mediated through its proapoptotic effects, which can occur in a p53-dependent or -independent manner (21,22) and involve its ability to induce a caspase cascade that includes caspase-1 (20,22), caspases-3/7 (20,23), caspase-8 (24) and/or Fas ligand (25). Caspases are known to affect antiestrogen responsiveness (26). Lower levels of IRF-1 protein have been reported in high-grade ductal carcinoma *in situ* or invasive ductal carcinoma of the breast when compared with adjacent normal breast epithelium (27).

The NFκB p50/p65 heterodimer complex comprises two homologous proteins encoded by different genes; the p105 precursor of p50 (NFκB1) is on chromosome 4, while the p65 (RelA) gene is on chromosome 11. The predominant form in human breast cancer cell lines is NFκB (p50/p65); another member of the family (p52) is also expressed in some breast cancers (28). NFκB (p50/p65) is implicated in several critical cellular functions including cell survival (29); these functions are often cell context specific (30). We have shown differential expression and activation of NFκB expression with both acquired antiestrogen resistance (11) and estrogen independence in breast cancer cells (13). Other studies also show increased expression of NFκB in endocrine-resistant breast cancer cell lines (31,32). We have begun to establish the functional relevance of these observations. For example, estrogen-independent cells significantly up-regulate NFκB; when its inhibitor IκBα is overexpressed in these cells their xenografts regress upon estrogen withdrawal (13). Antiestrogen-resistant cells are more sensitive to growth inhibition by parthenolide, a small molecule inhibitor of NFκB, than their antiestrogen sensitive parental cells (11). Furthermore,

parthenolide can reverse the antiestrogen resistance phenotype and synergistically interact with antiestrogens *in vitro* (17).

As a member of the ATF/CREB transcription factor family that activate promoters containing specific cyclic AMP responsive elements (CRE) (33), hXBP-1 regulates the expression of several tissue-specific genes, including tissue inhibitor of metalloproteinases, osteopontin and osteocalcin (34). Potentially downstream of NFκB activation in some cells (35), hXBP-1 is associated with increased proliferation and reduced apoptosis (16), which implies a survival function. Changes in cAMP concentrations and CRE activation have been widely implicated in carcinogenesis and endocrine signaling, including affecting signaling from ERα and PgR (36). While the role of hXBP-1 in the normal/neoplastic breast has not been studied in detail, hXBP-1 is part of a cluster of genes associated with some ERα-positive breast tumors (37,38) and a recent study suggests it may be expressed in breast cancer cells (39). We have previously implicated increased expression of hXBP-1 in acquired antiestrogen resistance (11). More recently, we have shown the ability of hXBP-1 to induce an estrogen-independent phenotype and to confer antiestrogen resistance (unpublished data).

The oncogenic nucleolar phosphoprotein, NPM, is a DNA-binding protein (40) that inhibits the ability of the YY1 (41) and IRF-1 transcription factors to regulate gene expression (42). NPM also serves as a substrate for several important serine-threonine kinases, including protein kinase C (43), p34^{cdc2} kinase (44,45) and casein kinase II (46). Insulin, which is a major mitogen for breast cancer cells, also increases NPM phosphorylation (45). Overexpression of NPM is sufficient to transform NIH/3T3 fibroblasts (47), and chromosomal translocations fusing NPM to either an anaplastic lymphoma kinase (48) or the retinoic acid receptor-α (49) have been reported in some cancers. We have shown that NPM is induced by estradiol (14) and expressed at higher levels in estrogen-independent breast cancer cells (11); a putative estrogen responsive element in the NPM promoter has now been recently described (50). In breast cancer patients, auto-antibodies to NPM are lower in patients treated with the antiestrogen Tamoxifen and increase six months prior to recurrence (15). Of particular relevance is the reduced expression of IRF-1 and concurrent increased expression of NPM, an endogenous IRF-1 inhibitor, in antiestrogen-resistant breast cancer cells (11).

We have now measured the expression of IRF-1, NFκB (p65), hXBP-1 and NPM in breast cancer specimens from women diagnosed at our institution. Using tissue microarrays and immunohistochemistry, we asked if these proteins could be detected in breast cancer, whether their expression might be correlated with other known prognostic markers, and whether the four proteins are expressed in patterns consistent with their known functions and/or our putative gene expression network. We find several proteins to be either coexpressed or inversely expressed in patterns consistent with our network hypothesis.

Materials and methods

Tissue specimens. Tissue microarrays were constructed using fifty-four, untreated, primary breast cancer cases diagnosed

between 1998 and 1999 from the breast cancer tumor bank at the Lombardi Comprehensive Cancer Center Histopathology Shared Resource at Georgetown University Medical Center. The cases were initially selected to determine the number of cores from a tumor needed to give the same estimation of ER α and PgR positivity as the entire section (51). Hence, the proportion of steroid hormone receptor-positive specimens (81% ER α ; 44% PgR) are higher than might be expected from a random sampling of breast cancer cases. While we cannot exclude the possibility of some selection bias in these cases, this selection should have identified cases most relevant to our initial hypothesis, implicating the proteins of interest in endocrine responsiveness (52). Differentiation/nuclear grade (53), DNA index (54) and S Phase (55) were determined as previously described. The categories for each end-point in Table I were selected prior to data analysis and are consistent with other studies (56,57). All material and information was collected and used in accordance with approved Institutional Review Board protocols. Clinical-outcome data and additional prognostic marker data are not available for these cases; available data are shown in Table I.

Tissue microarrays. Tissue microarrays were constructed with a Beecher Instruments manual tissue arrayer (Beecher Instruments, Inc., Sun Prairie, WI) as previously described (58,59). The instrument punches holes in the recipient paraffin block and acquires tissue cores from the donor block. Briefly, a thin-walled needle with an inner diameter of 0.6 mm was held in an X-Y precision guide. The cylindrical sample was retrieved from the selected region in the donor block and extruded directly into the recipient block with defined array coordinates. A solid steel wire, which closely fits the tube, was then used to transfer the tissue cores into the recipient block. The transfer was made under direct visual control with a stereotactic microscope using an additional bright light source. This cycle was repeated to obtain the appropriate number of cores. An adhesive-coated tape system (Instrumedics, Inc., Hackensack, NJ) was then used to cut 5 μ m sections of the tissue microarray block. The microtome knife cut underneath tape placed over the block surface. Thin tissue sections adhered to the tape, which was then rolled on an adhesive-coated microscope slide to transfer the section onto the slide. For this study, tissue microarrays were built with 480 cores from fifty-four breast carcinomas. Regions of invasive carcinoma were marked on each hematoxylin-and-eosin-stained slide. Ten cores were made from these areas of the paraffin block for 42 cases; for 12 additional cases, five cores were made. Thus, either 10 or 5 cores represented each tumor.

Antibodies and immunohistochemistry. The following commercial antibodies were used: ER α (ER1D5, Immunotech) (60), erbB2 (CB11; Zymed, San Francisco, CA) (61), hXBP-1 (sc-7160; Santa Cruz); IRF-1 (sc-497; Santa Cruz) (27), and NF κ B p65 (sc-109; Santa Cruz) (28). The NPM monoclonal antibody was kindly donated by Dr P-K Chan (46). Tissue microarray sections were deparaffinized in two 5-min changes of xylene and rehydrated through graded alcohol to distilled water. Immunohistochemistry was performed by a standard biotin-streptavidin-horseradish peroxidase method (63,64). Briefly, microarrays were treated with 1% H₂O₂ in methanol

Table I. Patient/tumor characteristics.

	n	Range
Age	54	36-85 (56) ^a
<50 years	25	
≥50 years	29	
Tumor grade	49	1-3
Grade 1	13	
Grade 2	26	
Grade 3	10	
Tumor size	54	0.2-6.8 (1.35)
<2 cm	39	
≥2 cm	15	
Lymph nodes	40	0-4
Negative (0)	28	
Positive (≥1)	12	
DNA index	47	1-2.89
<1.5%	27	
≥1.5%	20	
S-phase	28	1.64-27 (5.09)
<5% (low)	14	
≥5% (high)	14	

^aValues in parentheses are median values.

for 30 min to block endogenous peroxidase activity. Before applying the primary antibody, microarrays were boiled for antigen retrieval in 10 mM citrate buffer (pH 6.0) for a total 10 min. Microarrays were washed in phosphate-buffered saline containing 3% biotinylated goat antiserum to the appropriate IgG and 0.3% Triton X-100 (pH 7.4) for 30 min. Subsequently, tissue microarrays were incubated with the primary antibody at a 1:500 dilution (or as appropriate for the antibody) in PBS for 48 h at 4°C. After several washes, microarrays were treated with the appropriate secondary antibody (1:800; Vector Laboratories, Burlingame, CA) for 2 h, followed by a 1 h incubation with streptavidin-peroxidase conjugate (Vector Laboratories). Antigen-antibody complex was visualized by incubation with the VIP Kit (DAB Kit; Vector Laboratories). Finally, microarrays were counter-stained with either methyl green or hematoxylin, mounted and examined. All immunostaining was first optimized in single tissue slides. Negative controls were obtained using a standard method where microarrays are processed as described above but without incubation with the appropriate primary antibody.

Data analysis. The level of specific immunostaining, as determined relative to negative controls, was measured as an ordinal variable according to the nominal scale 0, 1+, 2+, 3+; where 0 is undetectable, 1+ refers to weak (barely perceptible) staining, 2+ to moderate staining, and 3+ to strong staining.

For nuclear staining, the scale applied was 1 = 0-25% of nuclei with detectable staining, 2 = 26-50%, 3 = 51-75%, 4 = ≥76%. The average score for all cores representing a tumor was used for data analysis. The relationships among staining values for each protein were compared using Spearman's rank order correlation coefficient analysis. All statistical tests are two-sided. We considered comparisons where $p < 0.05$ to be statistically significant; estimates of $p \geq 0.05$ and $p \leq 0.10$ were considered to indicate borderline statistical significance and potential biological relevance; comparisons where $p > 0.10$ were considered to be insignificant.

Pairwise correlation analyses could not account for the possibility that the associations of IRF-1n or IRF-1c may confound each other, since the expression of these two IRF measures may be correlated. To address this issue, we applied a novel use of partial correlation coefficient analysis, the partial correlations being calculated as shown in Eq 1:

$$r_{xy.z} = \frac{r_{xy} - (r_{xz})(r_{yz})}{\sqrt{(1 - r_{xz}^2)(1 - r_{yz}^2)}} \quad \text{Eq 1}$$

Where $r_{xy.z}$ = the correlation coefficient between x and y while controlling for the correlations between x and z and between y and z.

Partial correlations are most widely applied in the analysis of small signaling networks of 3-5 variables, and allow an estimate of the correlation between two variables while controlling for a third, fourth and/or fifth. Since we make functional assessments based upon cellular location, the use of partial correlations appears reasonable in the context of IRF-1n and IRF-1c. For the correlations between IRF-1c or IRF-1n and age, ER, PgR, NFκB and hXBP-1 the partial correlations were calculated with either IRF-1c or IRF-1n as the controlling variable.

Results

ERα and PgR expression. Measurements of ERα and PgR expression are the most widely used predictive factors in directing breast cancer therapy. The specimens in this study were originally selected to study ERα and PgR expression (51) and these two proteins are coexpressed in a substantial proportion of breast cancers. The expression of ERα (81%) and PgR (44%) using our criteria (Table II), and their significant coexpression in the tumors used in this study (Table V; $p < 0.001$) implies that the samples are likely to be broadly representative of ERα-positive breast cancers and appropriate for exploring protein expression patterns in cases likely to be selected for endocrine therapy.

IRF-1 expression. As a putative tumor suppressor, we might expect activated IRF-1 protein to be in the nucleus (IRF-1n) and inactive protein to be in the cytosol (IRF-1c). Whether these relationships are true for the IRF-1 signals we have measured is not known but we might expect the inactive form to predominate. In this context, and consistent with its putative tumor suppressor activities, the primary form of IRF-1 in breast tumors in this study appears to be IRF-1c

Table II. Immunohistochemical staining scores of five proteins detected in the cytosol.

Score	ERα	PgR	NFκB	^b IRF-1c	hXBP-1
0	^a 9	22	2	0	1
1+	1	7	18	4	9
2+	5	3	14	21	27
3+	37	20	13	17	10
Total	52	52	47	42	47
^c Detected	81% (42/52)	44% (23/52)	57% (27/47)	90% (38/42)	79% (37/47)

^aValues represent the number of cases in each category; scoring categories are described in Materials and methods. ^bIRF-1c, IRF-1 cytoplasmic staining. ^cDetected, proportion of cases with weak or stronger cytosolic staining.

(Fig. 2A). Of the tumors, 90% express detectable (1+ and higher) IRF-1c in their neoplastic cells, almost half of which have 3+ IRF-1 staining in the cytosol. In contrast, only 51% of the tumors in our study express detectable IRF-1n in >50% of the tumor cells and no tumors express IRF-1n in >75% of cells (Tables II and III). While 98% of the specimens express both detectable IRF-1c and IRF-1n, only 2% express IRF-1c alone and none express only IRF-1n. The inverse relationship between IRF-1n and IRF-1c ($p = 0.088$), while of borderline statistical significance, suggests that some breast tumors may differentially regulate the activation state of IRF-1 (Table V). This potential correlation raises the possibility that some associations implicating IRF-1c or IRF-1n may be confounded by the effect of the other. Our observations also are broadly consistent with a study reporting higher levels of IRF-1 protein in adjacent normal breast epithelium when compared with high-grade ductal carcinoma *in situ* or lymph node-positive invasive ductal carcinoma of the breast (27).

NFκB expression. We measured NFκB p65 expression, which is the predominant form of NFκB in human breast cancer cells (28) and the form associated with both estrogen independence (13) and acquired antiestrogen resistance (11). While active in breast cancer cell lines, NFκB p65 has been reported as being cytosolic (potentially inactive) whereas NFκB p50 has been reported to be primarily nuclear (active) in a prior study of $n = 17$ breast tumors (28). While the pattern of NFκB p65 staining is broadly consistent with this observation in many of our breast tumors (Fig. 2C), we found 57% of the tumors to express detectable (2+ or stronger) NFκB in their neoplastic cells (Table II).

hXBP-1 expression. Increased expression of hXBP-1, a nuclear transcription factor that activates cyclic AMP responsive elements (33), is associated with some forms of acquired antiestrogen resistance (11). hXBP-1 expression is detected in 79% of the breast tumors in this study (Table II), with the strongest staining seen in the cytosol (Fig. 2E). This observation

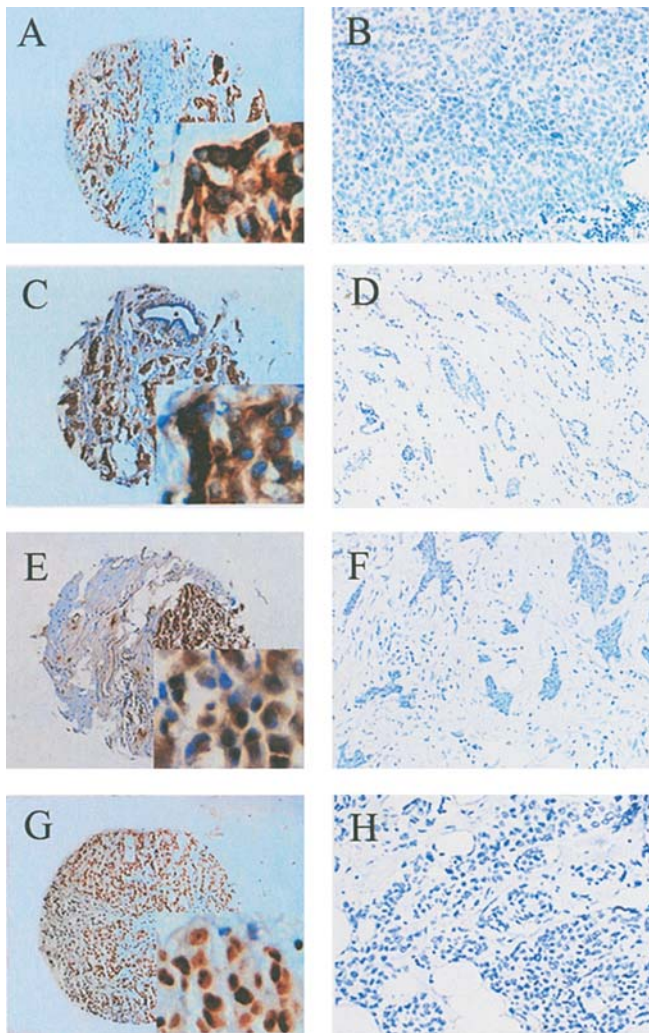


Figure 2. Representative immunostaining of IRF-1, NFκB p65, hXBP-1 and NPM in breast cancer. (A) IRF-1 staining where the inset shows typical patterns of cytosolic and nuclear staining; (B) Control for IRF-1 staining; (C) NFκB p65 staining where inset shows the primarily cytosolic staining pattern; (D) Control for NFκB p65 staining; (E) hXBP-1 staining where inset shows the primarily cytosolic staining pattern; (F) Control for hXBP-1 staining; (G) NPM staining where the inset shows the nuclear staining pattern for NPM; (H) Control for NPM staining. Figures of tissue microarray cores are at x10 magnification; inset at x100 magnification; control at x40 magnification.

is consistent with a small study of hXBP-1 expression in primary breast cancers (n=11) and breast cancer cell lines (n=5). In this recent study, expression was detected in all tumors and cell lines studied but hXBP-1 was almost undetectable in non-cancerous breast tissue (33).

NPM expression. NPM is a nucleolar phosphoprotein that is induced by estradiol (14) and expressed at higher levels in breast cancer cells with acquired antiestrogen resistance (11). In breast cancer patients, autoantibodies to NPM increase six months prior to recurrence and are lower in patients treated with TAM (15). Consistent with its nucleolar localization in cell culture, NPM staining is strongly nuclear in breast tumors (Fig. 2G). Of the breast cancers in this study, 95% express NPM in >50% of their neoplastic cell nuclei, the majority expressing NPM in >75% of their cells (Table II).

Table III. Immunohistochemical nuclear staining scores of IRF-1 and NPM.

Score	^b IRF-1n	NPM
1	^a 2	1
2	22	1
3	25	3
4	0	39
Total	49	44
^c >50%	51% (25/49)	95% (42/44)

^aValues represent the number of cases in each category; scoring categories are described in Materials and methods. ^bIRF-1n, IRF-1 nuclear staining. ^c>50%, proportion of cases where data are available that exhibit >50% of cell nuclei staining positive relative to the negative controls (NPM and IRF-1n are data for nuclear staining).

Correlation among proteins and patient/tumor characteristics. Several correlations among existing prognostic markers are known and are apparent in our data set (Table IV). Both PgR-positive (p=0.03) and ERα-positive tumors (borderline) are associated with a greater degree of differentiation and better prognosis (65). Borderline relationships between DNA index and both PgR-positivity and ERα-positivity (inverse correlation), and between NFκB and S phase (direct correlation) are also evident. A higher incidence of ERα-positive tumors is seen in older women (66) but our study was probably underpowered to detect this relationship. Nonetheless, the significant association between IRF-1c and age (Table IV; p=0.034) and the potential association between IRF-1c and ERα (p=0.079), may reflect the underlying relationship between ERα and age. We found no other associations among IRF-1, NFκB, hXBP-1 and NPM with either tumor grade, tumor size, DNA index, lymph node status, or S-phase fraction.

Correlation among protein expression patterns. Expression of several of the four proteins is correlated in breast tumors. Since our study is limited in size and power, we present those associations that reach conventional statistical significance and those where the association is of borderline statistical significance but of potential biological relevance. The data in Table V show coexpression of ER and IRF-1c (borderline), PgR and IRF-1n (p=0.014), IRF-1c and hXBP1 (p=0.001), IRF-1c and NFκB (p=0.002), and hXBP-1 and NFκB (0.018). Inverse correlations were seen between NPM and erbB2 (not shown; p=0.016), IRF-1n and NFκB (p=0.034), IRF-1n and IRF-1c (borderline), and IRF-1n and hXBP-1 (borderline). We estimated the partial correlations for each IRF-1n and IRF-1c correlation of interest; no effect is present when the sign and magnitude of the partial correlation coefficient is comparable to the original correlation coefficient. In each case, the partial coefficients were very similar to the original coefficients and shared the same sign. Hence, IRF-1c and IRF-1n are not antecedent, intervening, or suppressing variables

Table IV. Correlation among proteins and patient/tumor characteristics.

	ERα	PgR	IRF-1c	IRF-1n	NFκB	hXBP-1	NPM
Age	-	-	P=0.034 (r=0.28)	-	-	-	-
Tumor grade	P=0.067 (r=0.22) ^a	P=0.028 (r=0.28)	-	-	-	-	-
Tumor size	-	-	-	-	-	-	-
Lymph nodes	-	-	-	-	-	-	-
DNA index	P=0.077 (r=0.22)	P=0.086 (r=-0.21)	-	-	-	-	-
S phase	-	P=0.016 (r=0.41)	-	-	P=0.062 (r=0.33)	-	-

^aValues in parentheses are Spearman rank correlation coefficients. Comparisons where $p < 0.05$ are statistically significant; estimates of $p \geq 0.05$ and $p \leq 0.10$ are considered of borderline statistical significance and of potential biological relevance; comparisons where $p > 0.10$ were considered to be insignificant.

Table V. Correlation among protein expression patterns.

	ERα	PgR	^b IRF-1c	IRF-1n	NFκB	hXBP-1
PgR	P<0.001 (r=0.46) ^a	1				
IRF-1c	P=0.079 (r=0.23)	-	1			
IRF-1n	-	P=0.014 (r=0.32)	P=0.088 (r=-0.21)	1		
NFκB	-	-	P=0.002 (r=0.44) (r=0.42) ^c	P=0.034 (r=-0.27) (r=0.22) ^c	1	
hXBP-1	-	-	P=0.001 (r=0.49) (r=0.40) ^c	P=0.082 (r=-0.21) (r=-0.23) ^c	P=0.018 (r=0.31)	1
NPM	-	-	-	-		-

^aValues in parentheses are Spearman rank correlation coefficients; comparisons where $p < 0.05$ are statistically significant; estimates of $p \geq 0.05$ and $p \leq 0.10$ are considered of borderline statistical significance and of potential biological relevance; comparisons where $p > 0.10$ were considered to be not significant. ^bIRF-1c, cytoplasmic staining; IRF-1n, nuclear staining. ^cValues in parentheses are the estimated partial correlation coefficients. Use of partial correlation coefficients in networks can be found in De la Fuente, *et al*: Bioinformatics 20: 3565-3575, 2004.

for the correlations indicated, they exhibit respectively with hXBP-1 or NFκB (Table V).

Discussion

One approach to exploring the potential relevance of observations from experimental models is to determine

whether similar relationships may also arise in tumors from patients. While not directly informative in a mechanistic sense, identification of expression patterns in tumors that reflect patterns seen in xenografts and cell cultures can support mechanistic observations in these models. Furthermore, such studies may identify candidate biomarkers for further investigation. We have explored the expression levels

and patterns of coexpression of a subset of four genes (IRF-1, NF κ B p65, hXBP-1, NPM) implicated in endocrine resistance from our prior studies in experimental models (11-14).

Of the four genes we have previously implicated, IRF-1, NF κ B and hXBP-1 are transcription factors and NPM is a DNA-binding nucleolar phosphoprotein. Knowledge of a signal's cellular localization can provide mechanistic insight and all four proteins would be expected to exhibit some degree of nuclear staining that could reflect active protein. For example, NF κ B is maintained in the cytosol in an inactive state, complexed with members of the I κ B family (67). However, correctly identifying subcellular localization by immunohistochemistry can be confounded by fixation artifacts, leading to nuclear antigen redistribution during tissue processing. A fixation artifact is responsible for the apparent cytosolic localization of the NPM-anaplastic lymphoma kinase fusion antigen (68) but NPM staining is robust and primarily nuclear in our breast cancer specimens. In contrast, the activation state of hXBP-1 and NF κ B (p65) is difficult to determine because the staining is primarily cytosolic and any weak nuclear staining was not sufficient for further analysis. In this study, we chose to focus on the localization of IRF-1, which exhibits readily detectable nuclear and cytosolic staining patterns that appear inversely correlated (borderline; $p=0.088$). Furthermore, these patterns of staining for IRF-1 also make biological sense when considered in the context of putative active (nuclear) and inactive (cytosolic) states (see below).

Since there are only very limited published data on the expression of IRF-1, NF κ B, hXBP-1 and NPM in breast cancer, we first determined whether we could detect these proteins and estimate the extent to which they are expressed in this series of predominately ER+ breast tumors. All four proteins are detectable in the cases used in this study; NPM expression is detected in >25% of the neoplastic cells in almost all the breast cancers (95%). Consistent with recent reports, IRF-1 also is detected in breast tumors (90%) (27,69), as is hXBP-1 (79%) (39). NF κ B (p65) expression is the least frequently detected among the four proteins yet is detectable in 57% of the tumors. Thus, these four proteins are present in high proportions of breast cancer and are candidate biomarkers that merit further evaluation as both independent biomarkers and as a possible panel to be concurrently measured.

We have previously hypothesized that the proteins of interest are associated with affecting endocrine responsiveness (6,11,12). Acquired antiestrogen resistance primarily occurs in tumors that continue to express sufficient levels of ER α to be considered ER α -positive. While the primary form of *de novo* endocrine resistance is the absence of both ER α and PgR, a significant proportion of *de novo*-resistant tumors also are ER α -positive (52). The functional importance of continued receptor expression in either acquired or *de novo* endocrine resistance is unclear, but we might expect to find some of our network members to be coexpressed in the breast tumors used in this study (6).

A significant positive association between IRF-1n and PgR ($p=0.014$) and a borderline positive association between ER α and IRF-1c ($p=0.079$) are evident. Those PgR-positive tumors that coexpress IRF-1n may have a better prognosis and/or a better response to antiestrogens. For example, we

have recently shown that the ability of the steroidal antiestrogen ICI 182,780 (Faslodex; Fulvestrant) to signal apoptosis is mechanistically related to its ability to regulate IRF-1 expression and function in breast cancer cells (12).

We could not confirm coexpression of ER α and hXBP-1 ($p=0.244$), an association predicted from hierarchical cluster analysis of cDNA expression microarray data from human breast tumors (37,38). Several explanations for this outcome are possible. The nature of the signals from gene expression microarrays that measure mRNA and tissue microarrays that measure protein are very different. It also is not clear how closely the levels of mRNA and protein are related for hXBP-1. Furthermore, some of the associations/relationships identified in gene expression microarray studies were found by simple hierarchical clustering and these may not be correct or complete. The use of these clustering methods to identify gene expression patterns from within the very high dimensional data spaces generated by gene expression microarrays has been seriously questioned (70,71).

hXBP-1 expression is positively correlated with IRF-1c expression ($p=0.001$) but inversely associated with IRF-1n (borderline; $p=0.082$). These observations suggest a balance between IRF-1's inhibitory activity and hXBP-1's mitogenic activity. For example, tumors where hXBP-1 activity predominates may have a poor prognosis and/or poor response to antiestrogens. Some antiestrogen-resistant cells exhibit down-regulated IRF-1 activation and up-regulated hXBP-1 activity (western; promoter-reporter data) (11,12).

Expression of hXBP-1 and NF κ B (p65) are positively correlated ($p=0.018$). If we assume that NF κ B is inactive because of its cytosolic location, the coexpression of hXBP-1 might compensate for any lack of NF κ B in affecting cell survival since both are antiapoptotic. However, hXBP-1 expression appears to be downstream of NF κ B, at least in plasma cell differentiation (35), implying a potential induction of hXBP-1 by NF κ B. If this occurs in breast cancer cells and NF κ B is active, as suggested by the potential correlation between NF κ B and S-phase ($p=0.062$), it may explain the coexpression of hXBP-1 and NF κ B in Table V. We also cannot exclude the possibility that NF κ B p50 and/or NF κ B p52 expression are activated and may compensate for any loss of NF κ B p65 activity (28).

IRF-1 and NF κ B proteins form heterodimers that can regulate gene expression and we might expect to find these coexpressed in the same tumors. We found a significant coexpression of NF κ B and IRF-1c ($p=0.002$) and an inverse association between IRF-1n and NF κ B ($p=0.034$). Where both proteins are primarily sequestered in the cytosol, the ability of IRF-1:NF κ B heterodimers to regulate gene transcription could be inhibited. Several genes regulated by these heterodimers are implicated in breast cancer, including RANTES (regulated upon activation, normally T-Expressed and presumably secreted) (72), VCAM-1 (vascular cell adhesion molecule-1) (73) and IL-6 (interleukin-6) (74). RANTES expression correlates with a poor prognosis in breast cancer (75). VCAM-1 is involved in angiogenesis and metastasis in breast tumors (76), and an autocrine production of IL-6 is associated with drug resistance in breast cancer cells (77). The inverse relationship between IRF-1n and NF κ B suggests that some tumors may have activated IRF-1 in the absence of

active NFκB; such tumors may have a good prognosis and/or be sensitive to antiestrogens.

We obtained limited expression data for erbB2 (not shown). We detected a significant inverse association between erbB2 and NPM ($p=0.016$), suggesting that the oncogenic properties of NPM may be important in erbB2 non-overexpressing breast tumors, which represent the majority of breast cancer. No association was seen between erbB2 and either IRF-1, hXBP-1 or NFκB.

The present study represents the first analysis of the coexpression patterns of a subset of genes associated with acquired endocrine resistance in breast cancer cells. We could not adequately assess the activation state of each of the proteins and clinical-outcome data are not available in this data set. Despite these limitations, the data clearly show that all four proteins are detectable in a high proportion of the breast tumors used in this study. The data are consistent with a role for IRF-1, NFκB, hXBP-1 and NPM and their interactions in breast cancer, and are broadly supportive of the proposed component of a larger signaling network as outlined in Fig. 1. Further analysis of the expression patterns of IRF-1, NFκB, hXBP-1 and NPM as potential biomarkers for further defining endocrine response profiles in some breast cancer patients is warranted.

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References

1. Early Breast Cancer Trialists' Collaborative Group: Tamoxifen for early breast cancer: an overview of the randomized trials. *Lancet* 351: 1451-1467, 1998.
2. Early Breast Cancer Trialists' Collaborative Group: Systemic treatment of early breast cancer by hormonal, cytotoxic or immune therapy. *Lancet* 399: 1-15, 1992.
3. Miller WR: Biological rationale for endocrine therapy in breast cancer. *Best Pract Res Clin Endocrinol Metab* 18: 1-32, 2004.
4. Clarke R and Br  nner N: Acquired estrogen independence and antiestrogen resistance in breast cancer: estrogen receptor-driven phenotypes? *Trends Endocrinol Metab* 7: 25-35, 1996.
5. Clarke R, Skaar TC, Bouker KB, Davis N, Lee YR, Welch JN and Leonessa F: Molecular and pharmacological aspects of antiestrogen resistance. *J Steroid Biochem Mol Biol* 76: 71-84, 2001.
6. Clarke R, Liu MC, Bouker KB, Gu Z, Lee RY, Zhu Y, Skaar TC, Gomez B, O'Brien K, Wang Y and Hilakivi-Clarke LA: Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene* 22: 7316-7339, 2003.
7. Clarke R, Brunner N, Katzenellenbogen BS, Thompson EW, Norman MJ, Koppi C, Paik S, Lippman ME and Dickson RB: Progression from hormone dependent to hormone independent growth in MCF-7 human breast cancer cells. *Proc Natl Acad Sci USA* 86: 3649-3653, 1989.
8. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME and Clarke R: Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res* 53: 283-290, 1993.
9. Brunner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME and Clarke R: MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant which retains sensitivity to the steroidal antiestrogen ICI 182,780. *Cancer Res* 53: 3229-3232, 1993.
10. Brunner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SA and Clarke R: MCF7/LCC9: an antiestrogen resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early crossresistance to the non-steroidal antiestrogen tamoxifen. *Cancer Res* 57: 3486-3493, 1997.
11. Gu Z, Lee RY, Skaar TC, Bouker KB, Welch JN, Lu J, Liu A, Zhu Y, Davis N, Leonessa F, Brunner N, Wang Y and Clarke R: Association of interferon regulatory factor-1, nucleophosmin, nuclear factor-kappaB and cyclic AMP response element binding with acquired resistance to faslodex (ICI 182,780). *Cancer Res* 62: 3428-3437, 2002.
12. Bouker KB, Skaar TC, Fernandez DR, O'Brien KA, Riggins RB, Cao D and Clarke R: Interferon regulatory factor-1 mediates the proapoptotic but not cell cycle arrest effects of the steroidal antiestrogen ICI 182,780 (Faslodex, Fulvestrant). *Cancer Res* 64: 4030-4039, 2004.
13. Pratt MA, Bishop TE, White D, Yasvinski G, Menard M, Niu MY and Clarke R: Estrogen withdrawal-induced NF-kappaB activity and bcl-3 expression in breast cancer cells: roles in growth and hormone independence. *Mol Cell Biol* 23: 6887-6900, 2003.
14. Skaar TC, Prasad SC, Sharareh S, Lippman ME, Brunner N and Clarke R: Two-dimensional gel electrophoresis analyses identify nucleophosmin as an estrogen regulated protein associated with acquired estrogen-independence in human breast cancer cells. *J Steroid Biochem Mol Biol* 67: 391-402, 1998.
15. Brankin B, Skaar TC, Brotzman M, Trock B and Clarke R: Autoantibodies to numatrin: an early predictor for relapse in breast cancer. *Cancer Epidemiol Biomarkers Prev* 7: 1109-1115, 1998.
16. Reimold AM, Etkin A, Clauss I, Perkins A, Friend DS, Zhang J, Horton HF, Scott A, Orkin SH, Byrne MC, Grusby MJ and Glimcher LH: An essential role in liver development for transcription factor XBP-1. *Genes Dev* 14: 152-157, 2000.
17. Riggins RB, Zwart A, Nehra R and Clarke R: The NFκB inhibitor parthenolide restores ICI 182,780 (Faslodex; Fulvestrant)-induced apoptosis in antiestrogen resistant breast cancer cells. *Mol Cancer Ther* 4: 33-41, 2005.
18. Taniguchi T, Lamphier MS and Tanaka N: IRF-1: the transcription factor linking the interferon response and oncogenesis. *Biochim Biophys Acta* 1333: M9-M17, 1997.
19. Tanaka N, Ishihara M and Taniguchi T: Suppression of c-myc or fosB-induced cell transformation by the transcription factor IRF-1. *Cancer Lett* 83: 191-196, 1994.
20. Bowie ML, Dietze EC, Delrow J, Bean GR, Troch MM, Marjoram RJ and Seewaldt VL: Interferon-regulatory factor-1 is critical for tamoxifen-mediated apoptosis in human mammary epithelial cells. *Oncogene* 23: 8743-8755, 2004.
21. Tanaka N, Ishihara M, Lamphier MS, Nozawa H, Matsuyama T, Mak TW, Aizawa S, Tokino T, Oren M and Taniguchi T: Cooperation of the tumour suppressors IRF-1 and p53 in response to DNA damage. *Nature* 382: 816-818, 1996.
22. Tamura T, Ishihara M, Lamphier MS, Tanaka N, Oishi I, Aizawa S, Matsuyama T, Mak TW, Taki S and Taniguchi T: An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. *Nature* 376: 596-599, 1995.
23. Sanceau J, Hiscott J, Delattre O and Wietzerbin J: IFN-beta induces serine phosphorylation of Stat-1 in Ewing's sarcoma cells and mediates apoptosis via induction of IRF-1 and activation of caspase-7. *Oncogene* 19: 3372-3383, 2000.
24. Suk K, Chang I, Kim YH, Kim S, Kim JY, Kim H and Lee MS: Interferon gamma (IFNgamma) and tumor necrosis factor alpha synergism in ME-180 cervical cancer cell apoptosis and necrosis. IFNgamma inhibits cytoprotective NF-kappa B through STAT1/IRF-1 pathways. *J Biol Chem* 276: 13153-13159, 2001.
25. Chow WA, Fang JJ and Yee JK: The IFN regulatory factor family participates in regulation of Fas ligand gene expression in T cells. *J Immunol* 164: 3512-3518, 2000.

26. Mandlekar S, Hebbar V, Christov K and Kong AN: Pharmacodynamics of tamoxifen and its 4-hydroxy and N-desmethyl metabolites: activation of caspases and induction of apoptosis in rat mammary tumors and in human breast cancer cell lines. *Cancer Res* 60: 6601-6606, 2000.
27. Doherty GM, Boucher L, Sorenson K and Lowney J: Interferon regulatory factor expression in human breast cancer. *Ann Surg* 233: 623-629, 2001.
28. Cogswell PC, Guttridge DC, Funkhouser WK and Baldwin AS Jr: Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. *Oncogene* 19: 1123-1131, 2000.
29. Bours V, Bentires-Alj M, Hellin AC, Viatour P, Robe P, Delhalle S, Benoit V and Merville MP: Nuclear factor-kappa B, cancer and apoptosis. *Biochem Pharmacol* 60: 1085-1089, 2000.
30. Voegel JJ, Heine MJ, Zechel C, Chambon P and Gronemeyer H: TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J* 15: 3667-3675, 1996.
31. Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ and Sledge GW: Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol* 17: 3629-3639, 1997.
32. Osipo C, Gajdos C, Liu H, Chen B and Jordan VC: Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 95: 1597-1608, 1997.
33. Clauss IM, Chu M, Zhao JL and Glimcher LH: The basic domain/leucine zipper protein hXBP-1 preferentially binds to and transactivates CRE-like sequences containing an ACGT core. *Nucleic Acids Res* 24: 1855-1864, 1996.
34. Clauss IM, Gravalles EM, Darling JM, Shapiro F, Glimcher MJ and Glimcher LH: *In situ* hybridization studies suggest a role for the basic region- leucine zipper protein hXBP-1 in exocrine gland and skeletal development during mouse embryogenesis. *Dev Dyn* 197: 146-156, 1993.
35. Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravalles EM, Friend D, Grusby MJ, Alt F and Glimcher LH: Plasma cell differentiation requires the transcription factor XBP-1. *Nature* 412: 300-307, 2001.
36. Cho H, Aronica SM and Katzenellenbogen BS: Regulation of progesterone receptor gene expression in MCF-7 breast cancer cells: a comparison of the effects of cyclic adenosine 3',5'-monophosphate, estradiol, insulin-like growth factor-I and serum factors. *Endocrinology* 134: 658-664, 1994.
37. West M, Blanchette C, Dressman H, Huang E, Ishida S, Spang R, Zuzan H, Olson JA Jr, Marks JR and Nevins JR: Predicting the clinical status of human breast cancer by using gene expression profiles. *Proc Natl Acad Sci USA* 98: 11462-11467, 2001.
38. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO and Botstein D: Molecular portraits of human breast tumours. *Nature* 406: 747-752, 2000.
39. Fujimoto T, Onda M, Nagai H, Nagahata T, Ogawa K and Emi M: Up-regulation and overexpression of human X-box binding protein 1 (hXBP-1) gene in primary breast cancers. *Breast Cancer* 10: 301-306, 2003.
40. Feuerstein N, Mond JJ, Kinchington PR, Hickey R, Karjalainen Lindsberg ML, Hay I and Ruyechan WT: Evidence for DNA binding activity of numatrin (B23), a cell cycle-regulated nuclear matrix protein. *Biochim Biophys Acta* 1087: 127-136, 1990.
41. Inouye CJ and Seto E: Relief of YY1-induced transcriptional repression by protein-protein interaction with the nucleolar phosphoprotein B23. *J Biol Chem* 269: 6506-6510, 1994.
42. Kondo T: Identification and analysis of IRF-1 binding protein. *Hokkaido Igaku Zasshi* 71: 509-516, 1996.
43. Beckmann R, Buchner K, Jungblut PR, Eckerskorn C, Weise C, Hilbert R and Hucho F: Nuclear substrates of protein kinase C. *Eur J Biochem* 210: 45-51, 1992.
44. Peter M, Nakagawa J, Doree M, Labbe JC and Nigg EA: Identification of major nucleolar proteins as candidate mitotic substrates of cdc2 kinase. *Cell* 60: 791-801, 1990.
45. Feuerstein N and Randazzo PA: *In vivo* and *in vitro* phosphorylation studies of numatrin, a cell cycle regulated nuclear protein, in insulin-stimulated NIH 3T3 HIR cells. *Exp Cell Res* 194: 289-296, 1991.
46. Chan PK, Liu QR and Durban E: The major phosphorylation site of nucleophosmin (B23) is phosphorylated by a nuclear kinase II. *Biochem J* 270: 549-552, 1990.
47. Kondo T, Minamino N, Nagamura-Inoue T, Matsumoto M, Taniguchi T and Tanaka N: Identification and characterization of nucleophosmin/B23/numatrin which binds the anti-oncogenic transcription factor IRF-1 and manifests oncogenic activity. *Oncogene* 15: 1275-1281, 1997.
48. Morris SW, Kirstein MN, Valentine MB, Dittmer K, Shapiro DN, Look AT and Saltman DL: Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science* 263: 1281-1284, 1994.
49. Redner RL, Rush EA, Faas S, Rudert WA and Corey SJ: The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion. *Blood* 87: 882-886, 1996.
50. Bourdeau V, Deschenes J, Metivier R, Nagai Y, Nguyen D, Bretschneider N, Gannon F, White JH and Mader S: Genome-wide identification of high-affinity estrogen response elements in human and mouse. *Mol Endocrinol* 18: 1411-1427, 2004.
51. Singh B, To L, Ossandon M, Tefft M and Liu A: Representation of the heterogeneity of breast cancer and hormone receptor expression in a tissue microarray. *Mod Pathol* 13: 225A, 2000.
52. Clarke R, Leonessa F, Welch JN and Skaar TC: Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev* 53: 25-71, 2001.
53. Elston CW and Ellis IO: Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19: 403-410, 1991.
54. Atkin NB: Modal deoxyribonucleic acid value and survival in carcinoma of the breast. *Br Med J* 271: 272, 1972.
55. Fossa SD, Thorud E, Vaage S and Shoaib MC: DNA cytometry of primary breast cancer. Comparison of microspectrophotometry and flow cytometry, and different preparation methods for flow cytometric measurements. *Acta Pathol Microbiol Immunol Scand* 91A: 235-243, 1983.
56. Schneeweiss A, Sinn HP, Ehemann V, Khbeis T, Neben K, Krause U, Ho AD, Bastert G and Kramer A: Centrosomal aberrations in primary invasive breast cancer are associated with nodal status and hormone receptor expression. *Int J Cancer* 107: 346-352, 2003.
57. Konecny G, Pauletti G, Pegram M, Untch M, Dandekar S, Aguilar Z, Wilson C, Rong HM, Bauerfeind I, Felber M, Wang HJ, Beryt M, Seshadri R, Hepp H and Slamon DJ: Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. *J Natl Cancer Inst* 95: 142-153, 2003.
58. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G and Kallioniemi OP: Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 4: 844-847, 1998.
59. Moch H, Schraml P, Bubendorf L, Mirlacher M, Kononen J, Gasser T, Mihatsch MJ, Kallioniemi OP and Sauter G: High-throughput tissue microarray analysis to evaluate genes uncovered by cDNA microarray screening in renal cell carcinoma. *Am J Pathol* 154: 981-986, 1999.
60. Ellis MJ, Coop A, Singh B, Mauriac L, Llombert-Cussac A, Janicik F, Miller WR, Evans DB, Dugan M, Brady C, Quebe-Fehling E and Borgs M: Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor- positive primary breast cancer: evidence from a phase III randomized trial. *J Clin Oncol* 19: 3808-3816, 2001.
61. Rhodes A, Jasani B, Anderson E, Dodson AR and Balaton AJ: Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: a comparative study involving results from laboratories in 21 countries. *Am J Clin Pathol* 118: 408-417, 2002.
62. Nozawa Y, van Belzen N, van der Made ACJ, Dinjens WNM and Bosman FT: Expression of nucleophosmin/B23 in normal and neoplastic colorectal mucosa. *J Pathol* 178: 48-52, 1996.
63. Harlow E and Lane D: Antibodies. A Laboratory Manual. CSH, Cold Spring Harbor, 1988.
64. Mayer RJ and Walker JH: Immunohistochemical procedures. In: *Immunochemical Methods in Cell and Molecular Biology*. Academic Press, London, pp259-281, 1987.
65. Allred DC, Harvey JM, Berardo M and Clark GM: Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 11: 155-168, 1998.

66. Tarone RE and Chu KC: The greater impact of menopause on ER- than ER+ breast cancer incidence: a possible explanation (United States). *Cancer Causes Control* 13: 7-14, 2002.
67. Tam WF and Sen R: IkappaB family members function by different mechanisms. *J Biol Chem* 276: 7701-7704, 2001.
68. Mason DY, Pulford KA, Bischof D, Kuefer MU, Butler LH, Lamant L, Delsol G and Morris SW: Nucleolar localization of the nucleophosmin-anaplastic lymphoma kinase is not required for malignant transformation. *Cancer Res* 58: 1057-1062, 1998.
69. Hoshiya Y, Gupta V, Kawakubo H, Brachtel E, Carey JL, Sasur L, Scott A, Donahoe PK and Maheswaran S: Mullerian inhibiting substance promotes interferon gamma-induced gene expression and apoptosis in breast cancer cells. *J Biol Chem* 278: 51703-51712, 2003.
70. Hinneburg A and Keim DA: Optimal grid-clustering: towards breaking the curse of dimensionality in high-dimensional clustering. In: *Proceedings of the 25th Conference on Very Large Databases*. Atkinson MP, Orłowska ME, Valduriez P, Zdonik SB and Brodie ML (eds). Morgan Kaufman, San Francisco, pp506-517, 1999.
71. Satagopan JM and Panageas KS: A statistical perspective on gene expression data analysis. *Stat Med* 22: 481-499, 2003.
72. Lee AH, Hong JH and Seo YS: Tumour necrosis factor-alpha and interferon-gamma synergistically activate the RANTES promoter through nuclear factor kappaB and interferon regulatory factor 1 (IRF-1) transcription factors. *Biochem J* 350: 131-138, 2000.
73. Neish AS, Read MA, Thanos D, Pine R, Maniatis T and Collins T: Endothelial interferon regulatory factor 1 cooperates with NF-kappa B as a transcriptional activator of vascular cell adhesion molecule 1. *Mol Cell Biol* 15: 2558-2569, 1995.
74. Sanceau J, Kaisho T, Hirano T and Wietzerbin J: Triggering of the human interleukin-6 gene by interferon-gamma and tumor necrosis factor-alpha in monocytic cells involves cooperation between interferon regulatory factor-1, NF kappa B and Sp1 transcription factors. *J Biol Chem* 270: 27920-27931, 1995.
75. Luboshits G, Shina S, Kaplan O, Engelberg S, Nass D, Lifshitz-Mercer B, Chaitchik S, Keydar I and Ben-Baruch A: Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. *Cancer Res* 59: 4681-4687, 1999.
76. Byrne GJ, Ghellal A, Iddon J, Blann AD, Venizelos V, Kumar S, Howell A and Bundred NJ: Serum soluble vascular cell adhesion molecule-1: role as a surrogate marker of angiogenesis. *J Natl Cancer Inst* 92: 1329-1336, 2000.
77. Conze D, Weiss L, Regen PS, Bhushan A, Weaver D, Johnson P and Rincon M: Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. *Cancer Res* 61: 8851-8858, 2001.