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CENP-F expression is associated with poor prognosis and chromosomal instability in patients with primary breast cancer

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Abstract

DNA microarrays have the potential to classify tumors according to their transcriptome. Tissue microarrays (TMAs) facilitate the validation of biomarkers by offering a high-throughput approach to sample analysis. We reanalyzed a high profile breast cancer DNA microarray dataset containing 96 tumor samples using a powerful statistical approach, between group analyses. Among the genes we identified was centromere protein-F (*CENP-F*), a gene associated with poor prognosis. In a published follow-up breast cancer DNA microarray study, comprising 295 tumour samples, we found that *CENP-F* upregulation was significantly associated with worse overall survival ($p < 0.001$) and reduced metastasis-free survival ($p < 0.001$). To validate and expand upon these findings, we used 2 independent breast cancer patient cohorts represented on TMAs. CENP-F protein expression was evaluated by immunohistochemistry in 91 primary breast cancer samples from cohort I and 289 samples from cohort II. CENP-F correlated with markers of aggressive tumor behavior including ER negativity and high tumor grade. In cohort I, CENP-F was significantly associated with markers of CIN including cyclin E, increased telomerase activity, c-Myc amplification and aneuploidy. In cohort II, CENP-F correlated with VEGFR2, phosphorylated Ets-2 and Ki67, and in multivariate analysis, was an independent predictor of worse breast cancer-specific survival ($p = 0.036$) and overall survival ($p = 0.040$). In conclusion, we identified CENP-F as a biomarker associated with poor outcome in breast cancer and showed several novel associations of biological significance.

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Keywords

breast cancer; DNA microarrays; tissue microarrays; prognosis; CENP-F; chromosomal instability

In a study by van't Veer *et al*. published in 2002, a 70-gene prognosis classifier was identified *via* DNA microarray analysis of primary breast cancer that could be used to predict metastatic potential.[1] This work received considerable attention worldwide, and formed the basis for a clinical trial assessing the utility of DNA microarray technology in guiding treatment decisions for breast cancer patients.[2] However, some concerns have been raised about the data analysis methods and sample distributions utilized for this study.[3-5] To address such concerns, we reanalyzed this key DNA microarray dataset, validated our findings in 2 independent patient cohorts, and used tissue microarray (TMA) technology to explore biomarker associations with other known tumor variables. For re-analysis, we used the supervised method of BGA, which is based on carrying out an ordination (*e.g.* principal component analysis) of groups of samples rather than of individual samples. We previously demonstrated the successful application of BGA to DNA microarray data, and identified clinically important genes that were missed in previous analyses.[6] In the present study, we trained and cross-validated a gene classifier which maximally discriminated between patients with a good or poor prognosis. *CENP-F* was among the genes highly expressed in breast tumors of patients with poor prognosis, a finding that we validated in a related DNA microarray dataset.[5] Since little is known about the function of *CENP-F* in cancer, we examined its association with other known tumor parameters. Finally, we used immunohistochemistry on TMAs from 2 independent primary breast cancer cohorts to validate CENP-F protein expression as a prognostic marker, and identified coexpressed proteins that indicate a possible functional role of CENP-F in breast cancer.

Material and methods

Public DNA microarray datasets

Tumor samples and patient characteristics were described previously.[1,5] The van de Vijver dataset,[5] contained 61 patients with lymph node-negative disease from the original van't Veer study.[1]

DNA microarray data analysis

For the van't Veer dataset,[1] the expression data arising from analysis of ~25,000 human genes in 78 samples was filtered according to the original criteria.[1] In brief, genes were excluded if they did not display at least a 2-fold difference in expression and a p value of less than 0.01 in more than 3 samples. BGA, using Correspondence Analysis to ordinate the good and poor prognosis groups,[6] was applied to the resulting dataset of ~5,000 genes and used to classify the remaining 19 test samples. The 96 pooled training and test samples were randomly recategorized into 77 training and 19 test samples. Sample 54 was removed from the analysis as it contained >20% missing values. The 96 samples were re-split 100 times and BGA performed at each iteration. The top 100 genes associated with good prognosis and the top 100 genes associated with poor prognosis were then selected. BGA was performed using the ADE4 module from Bioconductor (http://www.bioconductor.org). Analysis was

performed using the statistical package R (http://www.r-project.org); the relevant R scripts are available on request. Data was downloaded from http://microarray-pubs.stanford.edu/wound_NKI/explore.html. For the van de Vijver dataset,[5] *CENP-F* mRNA expression was categorized as negative/low, unchanged, or high expression relative to pooled cRNA from each patient sample, acting as reference cRNA. Tumor samples were classified according to *CENP-F* mRNA expression based on absolute expression analysis P values (alpha level of 0.05), following the method of Moody *et al*.[7]

Patients and tumour samples for TMA analysis

Patients from the 2 independent primary breast cancer cohorts used in this study have been described previously.[8] In brief, cohort I consisted of 114 patients diagnosed with primary invasive breast cancer in Northern Sweden during 1988–1991. Samples were available from 91 patients for analysis of CENP-F expression.

Cohort II consisted of 512 consecutive breast cancer patients diagnosed at the Department of Pathology, Malmö University Hospital, Sweden during 1988–1992. Samples were available from 289 patients for analysis of CENP-F expression. The 289 tumor samples had a higher proportion of larger ($p < 0.001$), ER-negative ($p < 0.001$), high grade tumours ($p < 0.001$) and node-positive patients ($p = 0.019$), when compared with the 223 missing samples. There was no significant difference in patient age ($p = 0.367$), histological type ($p = 0.494$) or PR status ($p = 0.204$) between available and unavailable samples. Ethical approval was obtained for the use of human tissue samples for research from the Review Boards at Umeå and Lund universities, respectively.

Construction of TMAs and immunohistochemistry

TMAs were prepared separately for each cohort as previously described.[9] The tissue was deparaffinised, rehydrated and microwave-treated for 10 min in citrate buffer (pH 6.0). For detection of CENP-F, we used a rabbit polyclonal antibody (Abcam, Cambridge, UK; ab5) at a dilution of 1:100. Antibody specificity was confirmed by comparing the immunohistochemical staining of cell lines with corresponding Western blot reactivity. Nuclear staining immunoreactivity was determined by estimating the percentage of distinctly positive tumor cell nuclei. Based on previous studies of CENP-F,[10] we used a 10% cut-off point to categorise CENP-F expression, where 0–9% = "<10%"; and 10–100% = "≥10%". The results were separately scored by 2 observers and results compared. Any discrepancies in scoring were rescored by both observers together and a consensus reached. Evaluation of Ki67, VEGF-A, VEGFR1, VEGFR2, p53, phospho-ERK 1/2 and phospho-Ets-2 has been described elsewhere.[8,11-13]

Cell culture

The breast cancer cell lines T47D, BT474 and MDA-MB-231 and SK-BR3 were obtained from the European Collection of Cell Cultures, Wiltshire, UK. T47D, BT474 and MDA-MB-231 cell lines were grown in DMEM (Sigma, MO) supplemented with 10% FCS (Invitrogen, CA), L-glutamine (2 μM), penicillin (50 IU/ml) and streptomycin sulphate (50 μg/ml). SK-BR3 cells were grown in McCoy's 5a Medium (Sigma, MO) supplemented with

10% FCS. Cells were maintained in humidified air with 5% CO_2. Metaphase-arrested cells were obtained by incubating cells in the presence of nocodazole (1 μM) for 16 h.

Cell line array

The breast cancer cell lines T47D, SK-BR3, BT474 and MDA-MB-231 were used to optimize the anti-CENP-F antibody for immunohistochemical analysis. Cell lines were fixed in PFA for 30 min and resuspended in 70% ethanol overnight before being embedded in paraffin and arrayed.

Western blotting

Cultured cells were washed in 10 ml PBS, harvested and lysed in RIPA buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS and protease inhibitor cocktail (Sigma, MO). Protein levels were determined using the bicinchoninic acid (BCA) method (Pierce, IL). Samples containing 30 μg of protein were separated on a 3–8% Tris-acetate gel (Invitrogen, CA) by SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, Immobilin P (Millipore, MA). Membranes were blocked in 5% non-fat milk for 1 h. CENP-F expression was detected using a rabbit polyclonal anti-human CENP-F antibody (1:1500, clone ab5 from Abcam). Membranes were washed and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit antibody (1:10,000; Promega, UK). Antigen-antibody complexes were detected using ECL Plus reagent (Amersham Biosciences, Buckinghamshire, UK). Expression of cyclin E was measured by Western blotting and densitometry and was described previously.[11,14]

TMA statistical analysis

The χ^2 test for trend, Fisher's exact and Mann–Whitney tests were used for comparison of CENP-F expression with all other known parameters. Kaplan–Meier plots were used for survival analysis and the curves compared using the log-rank test.[15] Cox proportional hazards regression was used to estimate proportional hazard ratios and conduct multivariate analyses. All calculations were performed with SPSS v11.0 (SPSS, IL).

Results

Identification of alternative candidate biomarkers for primary breast cancer following reanalysis of DNA microarray data

When implementing BGA, we used an identical filter criterion set by van't Veer *et al.* in their original analysis,[1] which reduced the number of genes from 25,000 to just over 5,000. The original 78 training breast tumor samples were initially used to identify discriminating genes, and the 19 test samples used for validation of the identified genes. The classification accuracy we achieved was comparable to the original analysis,[1] with 84% of the test set being ascribed to the correct prognosis group (data not shown).

To remove a possible training and test sample selection bias, we randomly recategorized patient breast tumor samples into training and test samples. By applying BGA iteratively, the classification accuracy ranged from 36 to 84% with a median classification accuracy of 68%

(Fig. 1). A discrimination score was calculated for each gene by averaging the contribution (or weight) of a gene in each BGA over 100 iterations. Thus, this approach should produce a more robust gene ranking, as genes with more discrimination power should re-occur more frequently at higher rankings. Each of the 5,000 genes was ranked according to its average BGA co-ordinate. Supplementary Tables SIa and SIb detail the top 100 genes associated with good prognosis and the top 100 genes associated with poor prognosis, respectively, identified using BGA. Genes were then categorised into gene ontology (GO) categories (Table SII and Figs. S1 and S2). Genes involved in the cell cycle ($p \leq 0.001$) and movement/motor activity ($p \leq 0.001$) were significantly over-represented while genes involved in development ($p \leq 0.001$), signal transduction ($p \leq 0.001$) and cell communication ($p \leq 0.034$) were significantly under-represented, in the poor prognosis group. Tables SIIIa and SIIIb detail the functional categories associated with each of the top 100 genes associated with good prognosis and the top 100 genes associated with poor prognosis.

Among the genes we identified as highly associated with poor prognosis was *CENP-F*, which encodes for a kinetochore-associated protein implicated in the regulation of cell division,[16,17] *S100A9*, previously associated with inflammation and more recently with tumor development and metastasis,[18-20] *survivin*, an inhibitor of apoptosis and mitotic regulator,[21] *cathepsin L2*, a cysteine protease,[22] *BUB1*, a checkpoint kinase regulating the anaphase promoting complex or cyclosome,[23] *carbonic anhydrase IX*, a hypoxia-regulated enzyme involved in tumor cell survival,[24] a neuropeptide, *CART*[25] and *adrenomedullin*, an angiogenic peptide.[26] Genes we identified as being highly associated with good prognosis included *ER*, *PR*, *keratin 18*, and *serpinA3*, a protease inhibitor,[27] as well as *lipophilin B* and *mammaglobin A*, which form a heterodimeric complex and are overexpressed in breast cancer, but whose function remains unknown.[28,29] A heatmap was generated that depicts the association of the prognostic genes we identified, with their respective class (Fig. 2). Her2 expression was not a predictor of outcome in our re-analysis and ranked half way through the 5,000 significant genes in the dataset analysed (data not shown).

CENP-F is associated with poor prognosis in a related primary breast cancer DNA microarray dataset

CENP-F ranked 66 out of 100 genes associated with poor prognosis, with only small differences in correlation with survival between genes. We analyzed the expression of *CENP-F* in a related primary breast cancer DNA microarray dataset derived from 295 breast tumors.[5] Tumor samples were classified according to *CENP-F* mRNA expression based on absolute expression analysis P values (alpha level of 0.05), following the method used by Moody *et al.*[7] We found that *CENP-F* mRNA was overexpressed in 63 (21%) of the 295 tumors, with 108 tumors showing decreased expression and 124 tumors had no change in expression, relative to reference RNA. High *CENP-F* expression was associated with increased tumor size ($p = 0.028$), high tumor grade ($p < 0.001$) and ER-negative tumors ($p < 0.001$) (Table I). In addition, *CENP-F* mRNA expression was related significantly to reduced overall survival ($p < 0.001$) and reduced metastasis-free survival ($p < 0.001$) (Fig. 3).

Expression of CENP-F in primary breast cancer

Our next aim was to validate our findings using CENP-F expression at the protein level. The specificity of the anti-CENP-F antibody was first established using Western blotting, in parallel with immunohistochemical analysis of formalin-fixed, paraffin-embedded cell lines mimicking the handling of the primary tumors. CENP-F is maximally expressed in the G2/M phase of the cell cycle.[16,17] Each of the 4 breast cancer cell lines used in the study were treated with the anti-mitotic agent, nocodazole, which arrests cells at the G2/M phase of the cell cycle. As shown in Figure 4*a*, the anti-CENP-F antibody detected a protein of ~350 kDa by Western blotting, with M-phase arrested, nocodazole-treated cells showing increased CENP-F expression when compared with untreated cells, as expected. Nuclear expression of CENP-F was detected by immunohistochemistry in a proportion of cells in each of the cell lines examined, in the absence of nocodazole (Fig. 4*b*).

CENP-F expression was then assessed in 2 different breast cancer cohorts (I and II) arranged in TMAs. For breast cancer cohort I, 90 (99%) out of 91 tumors available for analysis, expressed nuclear CENP-F in various amounts (Fig. 5). In cohort II, 289 samples were available for analysis and nuclear staining was seen in 206 (71%) out of 289 specimens. Patients from cohort I were significantly younger ($p = 0.007$) and tumor size was significantly larger ($p = 0.006$) than samples available for analysis from cohort II, which could contribute to the differences in the proportion of CENP-F-positive tumors between the cohorts. In addition, cohort I included, more ER negative breast carcinomas compared to cohort II, 29% *versus* 20%, but this difference was not statistically significant. There was no significant difference in grade, nodal status or PR status between the samples analyzed in cohorts I and II.

CENP-F protein expression correlates with clinico-pathological parameters in primary breast cancer

In patient cohorts I and II, we analyzed potential associations between CENP-F expression and known clinico-pathological parameters such as tumor size, tumor type, grade, hormone receptor status, patient age and the presence of lymph node metastases. We used a 10% cut-off point for CENP-F expression to categorize samples into groups, in accordance with previous studies.[10] CENP-F expression was associated with ER-negative tumors ($p = 0.028$) in cohort II, and with high grade tumors in both cohort I ($p = 0.002$) and cohort II ($p < 0.001$) (Table II). CENP-F expression was not associated with tumor size, patient age, lymph node status, histological type or PR status in either cohort.

CENP-F protein expression correlates with tumour biological parameters in primary breast cancer

CENP-F expression was associated with the proliferation marker Ki67 in cohort II (Table III; $p < 0.001$) but not in cohort I (Table III; $p = 0.198$). In addition, CENP-F expression was associated with markers of chromosomal instability (CIN) including cyclin E overexpression ($p = 0.021$), survivin nuclear expression (unpublished data; $p = 0.001$), *c-Myc* amplification ($p = 0.003$), increased telomerase activity ($p = 0.002$) and aneuploidy ($p = 0.025$) in cohort I, indicating a link between CENP-F and CIN in these tumors (Table III). VEGF-A was not associated with CENP-F expression in either cohort I ($p = 0.070$) or cohort II ($p = 0.959$).

However, in cohort II, significant associations were observed between CENP-F expression and tumor-specific VEGFR2 expression ($p = 0.001$) and phosphorylated Ets-2 ($p = 0.001$) but not with phosphorylated Erk1/2 ($p = 0.190$) (Table III). Finally, we found no association between CENP-F expression and tumor specific VEGFR1, p53 or Her2 overexpression (Tables II and III).

CENP-F protein expression correlates with clinical outcome in primary breast cancer

Clinical follow-up data were available for all patients in cohorts I and II.[8] Patient cohorts were analyzed separately. In agreement with previous findings,[10] we found that using a cut-off of 10% CENP-F expression best separated patients on the basis of survival. In cohort I, CENP-F expression showed a significant association with overall survival ($p = 0.05$; Fig. 6*a*). In cohort II, expression of CENP-F correlated significantly with both breast cancer specific survival ($p = 0.009$) and overall survival ($p = 0.04$) (Fig. 6*b* and 6*c*).

Patients from cohort I had overall survival information only, and patient numbers were too low to carry out multivariate Cox regression analysis. In a univariate analysis of cohort I, CENP-F expression was associated with worse overall survival, with this association approaching significance (HR, 2.03; 95% CI, 0.97–4.24; $p = 0.059$). Univariate and multivariate Cox regression analyses were conducted on cohort II (Table IV). CENP-F expression, ER and tumor size were all significantly associated with breast cancer-specific survival ($p = 0.011$; 0.006; 0.001, respectively) but patient age, VEGFR2 and phospho-Ets-2 were not ($p = 0.088$; 0.408 and 0.544, respectively). In a multivariate analysis including ER and tumor size, CENP-F expression was an independent predictor of breast cancer-specific survival ($p = 0.036$) (Table IV). Tumor grade was not included in the multivariate model as our data suggests that CENP-F is involved in CIN; thus, tumor grade may be on a causal pathway between CENP-F and survival and should, therefore, not be included in multivariate models with CENP-F.[30,31] Using a similar approach, we carried out univariate analysis for overall survival on CENP-F expression, ER, VEGFR2, phospho-Ets-2, tumor size and patient age (Table V). CENP-F expression, tumor size and patient age were significantly associated with overall survival in a univariate analysis ($p = 0.047$; <0.001; <0.001, respectively), and in a multivariate analysis CENP-F retained its prognostic significance ($p = 0.040$) together with patient age ($p < 0.001$) (Table V). Ki67 showed only borderline significant association with overall survival in cohort II ($p = 0.05$) and was not significant when added to multivariate models for breast cancer specific or overall survival (data not shown).

Discussion

DNA microarrays offer new possibilities for the elucidation of individual genes and groups of genes that are preferentially expressed in tumor subgroups. The 70-gene prognosis classifier identified by van't Veer *et al.*[1] contained a large number of unknown or unexpected genes and none of the well-known prognostic markers in breast cancer such as ER, Her-2, uPA or PAI-1.[32] This dataset forms the basis of a clinical trial, which aims to validate the efficacy of using the identified classifier for tailoring of treatment options. However, the methodology used to obtain this gene signature has been criticized.[3,4]

Given the complexity of selecting a relatively small number of informative genes from the many thousands of genes represented on a DNA microarray, reanalysis of such data using alternative approaches to identify discriminating genes is warranted. Here, we used the statistical method of BGA, a powerful method for the analysis of cancer microarray data,[6] to reanalyze this breast cancer dataset from van't Veer *et al.*[1] Our reanalysis approach revealed genes involved in key processes such as checkpoint control, apoptosis and angiogenesis, most of which were previously unidentified in the original analysis.

The classification accuracy we achieved using the same training and test samples as van't Veer *et al.*[1] was comparable to published results, *i.e.* 84%. However, when training and test samples were selected randomly, the classification accuracy varied widely from a maximum accuracy of 84% to as low as 36%, with a median classification accuracy of 68%. Similar findings have been published by others,[3,4] suggesting a bias in the selection of the original training and test samples.

In our reanalysis of the van't Veer dataset,[1] *CENP-F* was among the genes that were highly associated with poor prognosis that could be studied at the protein level using TMAs. CENP-F is a cell cycle-regulated protein associated with kinetochores, the site at which chromosome-microtubule interactions are monitored and the source of checkpoint signals.[33] CENP-F is maximally expressed at the G2/M phase of the cell cycle[16,17] and has been implicated in kinetochore assembly and/or the spindle checkpoint.[34,35] More recently, CENP-F has been shown to play a central role in the recruitment of the checkpoint proteins, BubR1 and Mad1, resulting in a sustained checkpoint response.[36]

In a related DNA microarray dataset that we reanalyzed containing 295 breast tumor samples,[5] over-expression of CENP-F mRNA was associated with larger tumor size, as well as ER-negative, high grade tumors. *CENP*-F mRNA expression correlated significantly with worse overall survival and a decreased probability of remaining metastasis-free.

Two different primary breast cancer cohorts were used to further investigate the role of CENP-F. Two cohorts were analyzed, as each cohort has unique data available. CENP-F protein expression correlated with reduced breast cancer-specific survival and overall survival in both univariate and multivariate analyses. The strong correlation between CENP-F expression and breast cancer-specific survival highlights the usefulness of CENP-F as a breast cancer-specific marker of poor outcome. Our findings are in agreement with a previous report analyzing CENP-F expression and disease-free survival in node-negative breast cancer patients.[10]

In cohort I, parameters relating to cell cycle deregulation and CIN had previously been analyzed.[14,37] CENP-F expression was associated with cyclin E over-expression, survivin nuclear expression and *c-Myc* amplification. Cyclin E is involved in centro-some duplication leading to CIN,[38-40] while constitutive expression of cyclin E has been shown to result in CIN[41,42] and is associated with poor prognosis in breast cancer.[14] Survivin has been reported to activate the cyclin E/Cdk2 complex resulting in an accelerated S phase shift.[43] CENP-F expression was also associated with *c-Myc* amplification which has been shown to activate cyclin E/Cdk2, leading to cell cycle progression and proliferation.[44] In addition,

CENP-F expression correlated significantly with high telomerase activity. Telomerase activation is associated with telomere dysfunction, a major mechanism underlying CIN of human cancer.[45,46] Furthermore, a significant proportion of tumors over-expressing CENP-F were aneuploid, strengthening the relation between CENP-F expression and markers of CIN. Additional studies, including FISH analysis, will determine if these associations have functional significance. FISH analysis could not be performed in this study because of insufficient sample availability

While tumor VEGF-A expression did not correlate with CENP-F expression in either patient cohort I or II, we found a significant correlation between tumor cell VEGFR2 expression and CENP-F in cohort II. CENP-F is a phosphoprotein but it is not known which kinases target CENP-F for phosphorylation or the role of phosphorylation in CENP-F regulation. It is tempting to speculate that CENP-F may be a target for phosphorylation through cyclin E or VEGFR2, as CENP-F is significantly associated with expression of both of these proteins. However, further studies will need to be carried out to establish a functional link.

In line with other publications, CENP-F was associated with proliferation[47-49] and ER negativity[5,10] in cohort II. Furthermore, CENP-F expression was associated with the transcription factor phospho-Ets-2. Ets-2 expression in breast cancer may be linked to proliferation,[8] however, the downstream target genes are unknown. CENP-F regulates gene transcription and proliferation through association with the transcription factor ATF4.[50] Our results suggest that CENP-F may be a potential candidate for Ets-2 co-transcriptional regulation.

CENP-F is a farnesylated protein and is targeted by farnesyl transferase inhibitors (FTIs)[51,52] resulting in CENP-F inactivation. Originally generated to inhibit oncogenic RAS, FTIs are effective anti-neoplastic agents. It is now becoming apparent that RAS is not the only target of FTIs; however, the role of other molecular targets and their mechanism of action remains elusive.[53] FTIs have been shown to be effective in clinical trials of patients with metastatic breast carcinoma, especially in Her2 positive patients[54,55] and CENP-F-positive breast cancer has a pathologic response to preoperative chemotherapy.[56] FTI-sensitive cells pause at the G2/M phase of the cell cycle[51,57] and have misaligned chromosomes,[58] similar to cells depleted of CENP-F by RNAi.[59,60] The anti-neoplastic activity, involving inhibition of proliferation and, apoptosis may be partly due to CENP-F inhibition. Thus CENP-F may be an important, clinically significant target in breast cancer and CENP-F farnesylation a useful biomarker of tumor response.

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Abbreviations

BGA	between group analysis
CENP-F	centromere protein F
CIN	chromosomal instability
ER	oestrogen receptor
ERK 1/2	extracellular signal-regulated kinase 1/2
FCS	foetal calf serum
FISH	fluorescence in situ hybridization
GO	gene ontology
Her2	human epidermal growth factor receptor 2
MMP-9	matrix metalloproteinase-9
PAI-1	plasminogen activator inhibitor 1
TMA	tissue microarray
uPA	urokinase plasminogen activator
VEGF-A	vascular endothelial growth factor-A
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2

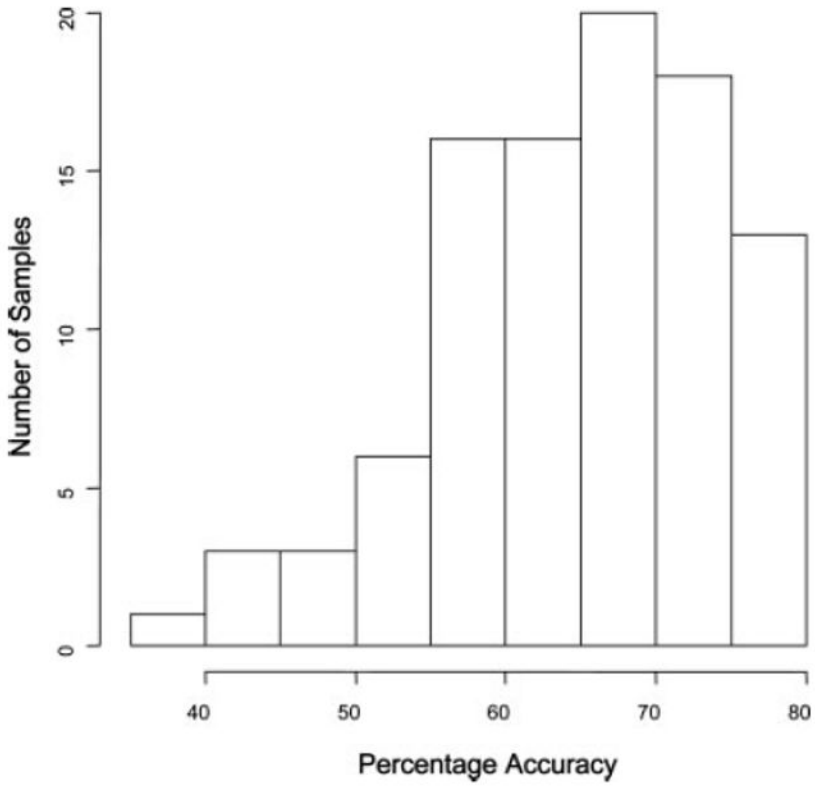

Figure 1.

Histogram of percent classification accuracy using randomly selected training and test samples. BGA was applied iteratively on 96 samples that were randomly split 100 times into 77 training and 19 test samples. The accuracy of the classification of the test samples was recorded at each iteration and plotted on a graph. The training and test data contained 5,000 genes in each case.

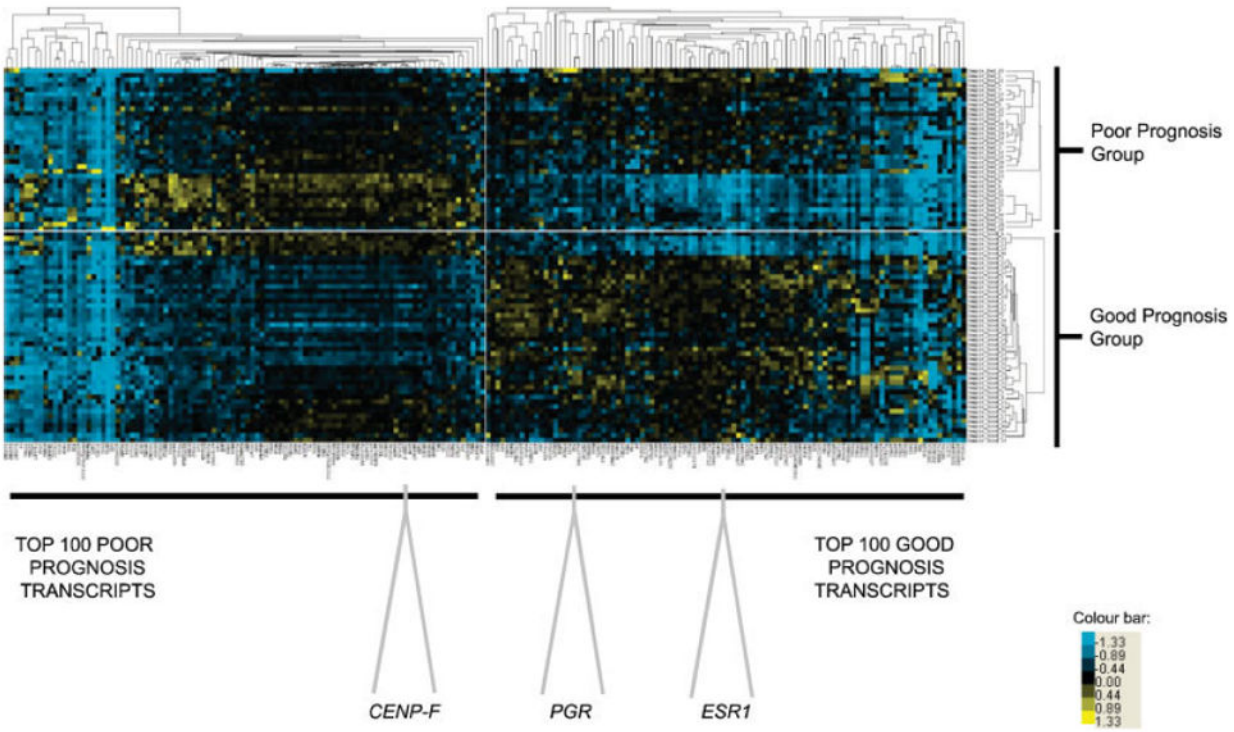

Figure 2.

Heatmap of top 100 genes associated with good prognosis and the top 100 genes associated with poor prognosis. Each row represents a tumor and each column a gene transcript. PGR = the HUGO gene symbol for the progesterone receptor, ESR1 = the HUGO gene symbol for the oestrogen receptor. The colour bar is in a log10 scale. Tumours and genes are clustered according to expression levels. Hierarchical clustering analysis was performed using average linkage and the Euclidean distance metric.

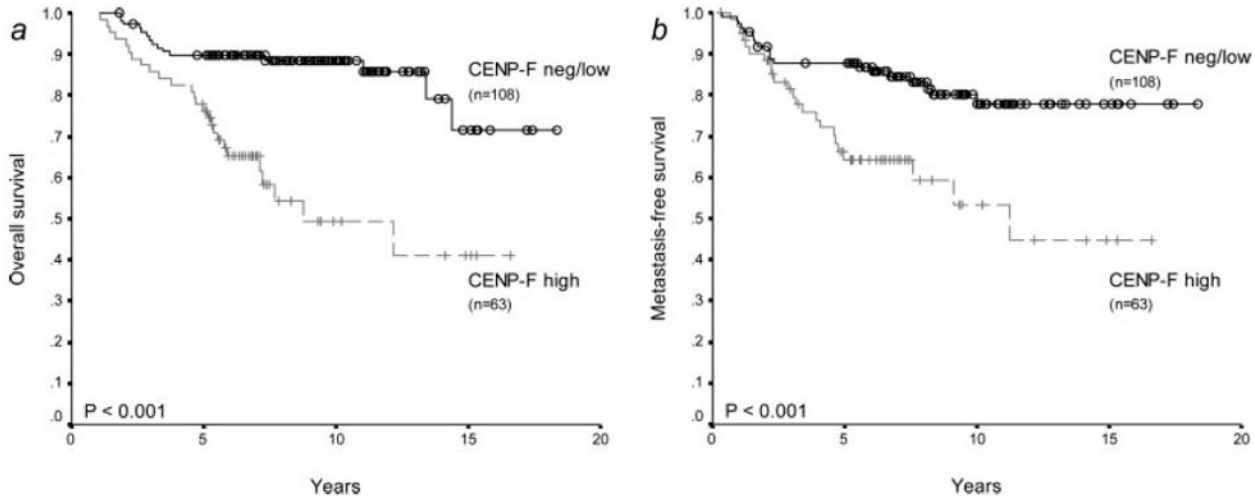

Figure 3.

Kaplan-Meier analysis of (*a*) overall survival and (*b*) metastasis-free survival of 295 patients from the van de Vijver study. Patients are stratified on the basis of low or high CENP-F expression, as previously measured by DNA microarray analysis.

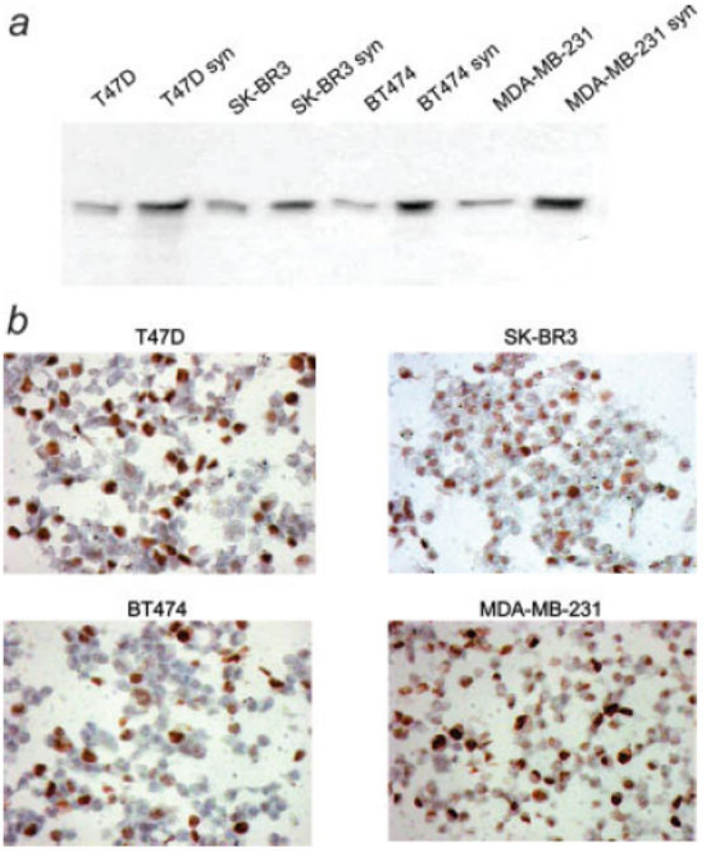

Figure 4.

Validation of the anti-CENP-F antibody using Western blotting and immunohistochemistry. (*a*) Protein extracts from T47D, SK-BR3, BT474, and MDA-MB-231 cells gave a single distinct band of approximately 350 kDa via Western blot analysis. (*b*) CENP-F was expressed in a proportion of cells in each of the cell lines examined by immunohistochemistry, in the absence of nocodazole. Syn = cells synchronised at mitosis using the mitotic inhibitor, nocodazole.

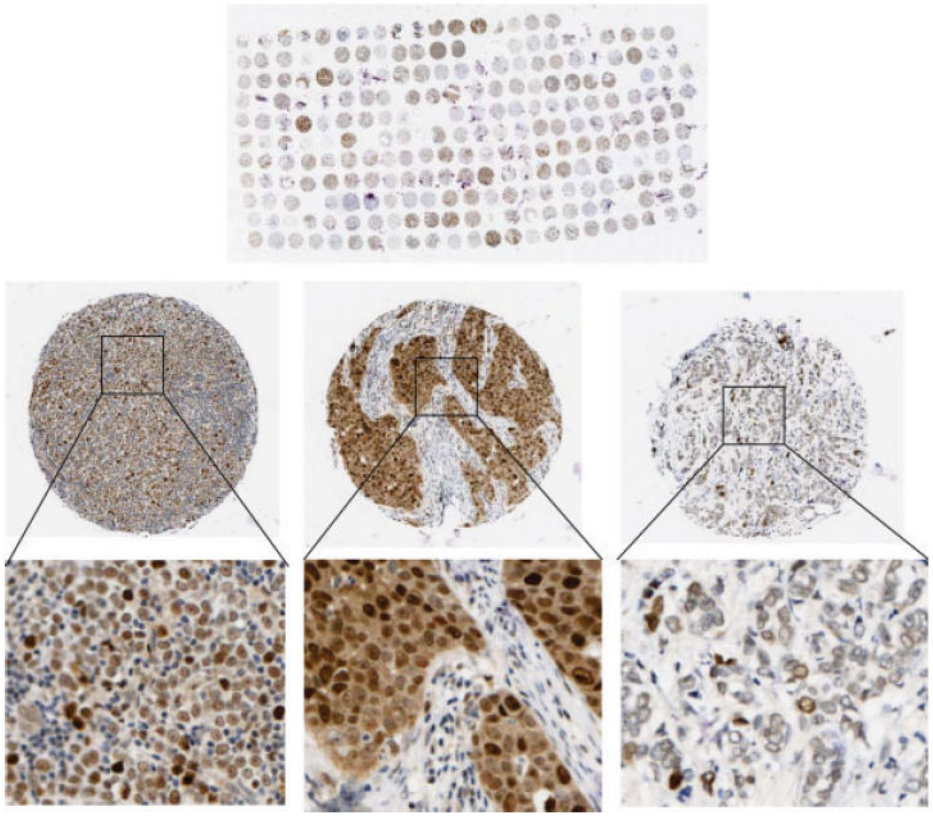

Figure 5.

A breast cancer TMA stained immunohistochemically with the anti-CENP-F antibody. CENP-F expression is indicated by brown staining. Nuclear staining of different intensities and fractions were observed only in tumor cells.

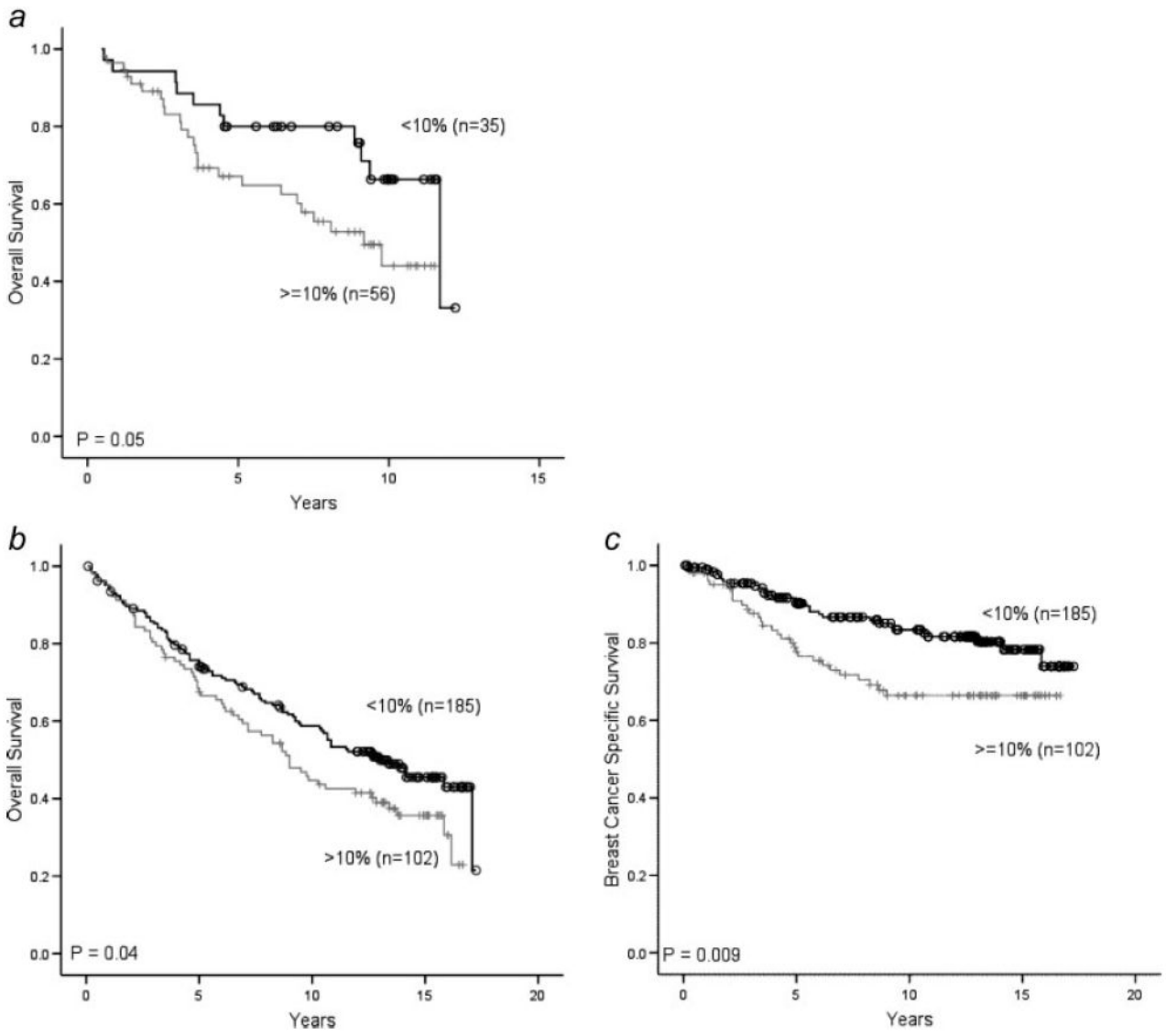

Figure 6.

Kaplan-Meier analysis of patients with invasive breast cancer stratified by CENP-F expression. (*a*) Overall survival of 91 patients from cohort I, (*b*) overall survival of 261 patients from cohort II and (*c*) breast cancer-specific survival in 261 patients from cohort II. A cut-off point of 10% positive nuclei was used. The P values were calculated using the log-rank test.

TABLE I

ASSOCIATION BETWEEN CENP-F EXPRESSION IN THE VAN DE VIJVER DNA MICROARRAY DATASET, AND CLINICOPATHOLOGICAL PARAMETERS

Variable	CENP-F (negative/low) (*n* = 108)	CENP-F (high) (*n* = 63)	*P* value (χ^2 test)
Age			
Median (range)	43 (32–52)	45 (26–52)	
<Median (26–44)	49 (45)	33 (52)	0.376
>Median (44–53)	59 (55)	30 (48)	
Tumor size (mm)			
Median (range)	20 (2–0)	23 (10–2)	
T1 (1–20)	65 (60)	27 (43)	0.028
T2 (>20)	43 (40)	36 (57)	
NHG[1]			
1	45 (42)	6 (10)	<0.001
2	38 (35)	18 (29)	
3	25 (23)	29 (61)	
Lymph-node status			
Negative	65 (60)	35 (56)	0.553
Positive	43 (40)	28 (44)	
ER status			
ER−	13 (12)	25 (40)	<0.001
ER+	95 (88)	38 (60)	

CENP-F expression levels were unchanged in 124 tumors.

[1]NHG = Nottingham histological grade.

TABLE II

ASSOCIATION BETWEEN CLINICOPATHOLOGICAL PARAMETERS AND CENP-F EXPRESSION IN COHORTS I AND II

Variable	Cohort I			*p-value*	Cohort II			*p-value*
	N	**CENP-F < 10%**	**CENP-F ≥ 10%**		***n***	**CENP-F < 10%**	**CENP-F ≥ 10%**	
Age	91			0.063[1]	289			0.375[1]
n		35	56			186	103	
Median (range)		56 (33–82)	62 (30–84)			65 (28–92)	64 (27–96)	
Tumour size (mm)	86			0.212[1]	263			0.660[1]
n		35	51			186	103	
Median (range)		20 (10–60)	25 (12–100			19 (1–100)	20 (0–100)	
Histological type	91			0.725[2]	242			0.581[2]
Ductal		33 (94)[3]	49 (88)[3]			122 (72)[3]	70 (73)[3]	
Lobular		2 (6)	3 (5)			29 (17)	12 (13)	
Medullary		0	2 (3.5)			7 (4)	8 (8)	
Tubular		0	0			6 (3.5)	4 (4)	
Mucinous		0	2 (3.5)			6 (3.5)	2 (2)	
Lymph-node status	84			0.660[2]	255			0.083[2]
Negative		19 (56)	25 (50)			104 (63)	46 (51)	
Positive		15 (44)	25 (50)			61 (37)	44 (49)	
ER status[4]	90			0.059[2]	282[5]			0.028[2]
ER−		6 (17)	20 (36)			29 (16)	27 (27)	
ER+		29 (83)	35 (64)			154 (84)	72 (73)	
PR status[5]	84			0.512[2]	255			0.289[2]
PR–		19 (56)	24 (48)			61 (38)	42 (45)	
PR+		15 (44)	26 (52)			101 (62)	51 (55)	
NHG	91			0.002[6]	288			<0.001[6]
1		17 (48)	19 (34)			37 (20)	12 (12)	
2		9 (26)	5 (9)			92 (50)	30 (29)	
3		9 (26)	32 (57)			57 (30)	60 (59)	

NHG, Nottingham histological grade.

[1] Mann-Whitney test.

[2] Fisher's exact test.

[3] Values in parentheses indicate percentages.

[4] Measured by enzyme linked immunosorbant assay.

[5] Measured by immunohistochemistry.

[6] χ^2 test for trend.

TABLE III

ASSOCIATION BETWEEN TUMOUR BIOLOGICAL PARAMETERS AND CENP-F EXPRESSION IN COHORTS I AND II

Variable (*n*)	CENP-F <10%	CENP-F ≥10%	*p value*
Cohort I			
VEGF-A[1] (89)			0.070[2]
Low	5 (14)	3 (5.6)	
Intermediate	23 (66)	29 (53.7)	
High	7 (20)	22 (40.7)	
Cyclin E[3] (91)			0.021[2]
Low	29 (83)	33 (59)	
High	6 (17)	23 (41)	
Telomerase activity[4] (86)			0.002[5]
Median	0.81	3	
Range	0–54.7	0–153	
Myc amplification[6] (71)			0.003[7]
Low	27 (96)	29 (67)	
Intermediate/High	1 (4)	14 (33)	
Ploidy (85)			0.025[7]
Diploid	19 (61)	19 (35)	
Aneuploid	12 (39)	35 (65)	
Survivin-nuclear[1] (91)			0.001[7]
Negative	7 (20)	5 (9)	
<50%	22 (63)	21 (37.5)	
>50%	6 (17)	30 (53.5)	
Her2[6] (88)			0.423[2]
Grade 0–2	29 (85)	42 (78)	
Grade 3	5 (15)	12 (22)	
p53 status[1] (89)			0.095[2]
p53 −	27 (82)	36 (64)	
p53 +	6 (18)	20 (36)	
Ki67[1] (89)			0.198[2]
≤10%	11 (32)	10 (18)	
>10%	23 (68)	45 (82)	
Cohort II			
VEGFR2[1] (238)			0.001[7]
Neg/ Low	76 (50)	23 (26)	
Intermediate	55 (37)	44 (51)	
High	20 (13)	20 (23)	
VEGF-A[1] (235)			0.959[7]

Variable (*n*)	CENP-F <10%	CENP-F ≥10%	*p value*
Neg/Low	54 (37)	35 (39)	
Intermediate	65 (44.5)	35 (39)	
High	27 (18.5)	19 (22)	
VEGFR1[1] (275)			0.338[7]
Neg/Low	28 (16)	15 (15)	
Intermediate	75 (42)	34 (35)	
High	74 (42)	49 (50)	
Phospho-Ets-2[1] (240)			0.001[7]
Negative	59 (37)	18 (18)	
Low	43 (27)	34 (35)	
Intermediate	41 (26)	24 (25)	
High	15 (10)	22 (22)	
Phospho-Erk 1/2[1] (231)			0.190[7]
Negative	66 (43)	32 (35.5)	
Low	43 (28)	17(30)	
Intermediate	29 (19)	13 (19)	
High	16 (10)	14 (15.5)	
Ki67[1] (260)			<0.001[7]
≤10%	76 (42)	15 (15)	
>10%	106 (58)	83 (85)	

Values inside parentheses indicate percentages.

[1]Measured by immunohistochemistry.

[2]Fisher's exact test.

[3]Measured by Western blotting and densitometry.

[4]Measured by the telomeric repeat amplification protocol.

[5]Mann-Whitney test.

[6]Measured by fluorescence in situ hybridisation.

[7]Chi-square test for trend.

TABLE IV

COX REGRESSION ANALYSIS FOR BREAST CANCER-SPECIFIC SURVIVAL IN COHORT II

Prognostic factor	Univariate			Multivariate		
	HR	95% CI	*p*-Value	HR[1]	95% CI	*p*-Value
CENP-F(<10% *vs.* ≥ 10%)	1.93	1.16–3.21	0.011	1.76	1.04–2.98	0.036
Tumour size (mm) (continuous)	1.01	1.01–1.02	0.001	1.01	1.00–1.02	0.029
ER (pos *vs.* neg)	0.49	0.29–0.81	0.006	0.54	0.31–0.97	0.037
Patient age (continuous)	0.99	0.97–1.01	0.088	n/a		
VEGFR2 (grade 3 *vs.* 0–2)	0.80	0.38–1.67	0.544	n/a		
Phospho-Ets-2 (grade 3 *vs.* 0–2)	0.79	0.39–1.59	0.513	n/a		

HR = hazard ratio, CI = confidence interval, n/a = Not applicable.

[1]Adjusted for all other variables indicated.

TABLE V

COX REGRESSION ANALYSIS FOR OVERALL SURVIVAL IN COHORT II

Prognostic factor	Univariate			Multivariate		
	HR	95% CI	*p*-Value	HR[1]	95% CI	*p*-Value
CENP-F(<10% *v* ≥ 10%)	1.38	1.01–1.90	0.047	1.40	1.02–1.92	0.040
Tumour size (mm) (continuous)	1.01	1.01–1.01	<0.001	1.00	0.997–1.01	0.261
Patient age (continuous)	1.06	1.05–1.07	<0.001	1.05	1.03–1.06	<0.001
ER (pos *vs.* neg)	0.73	0.52–1.01	0.057	n/a		
VEGFR2 (grade 3 *vs.* 0–2)	1.19	0.81–1.76	0.380	n/a		
Phospho-Ets-2 (grade 3 *vs.* 0–2)	1.06	0.72–1.56	0.780	n/a		

HR = hazard ratio, CI = confidence interval, n/a = Not applicable.

[1]Adjusted for all other variables indicated.