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PRECLINICAL STUDY

Transferrin receptor (CD71) is a marker of poor prognosis in breast cancer and can predict response to tamoxifen

Hany Onsy Habashy · Desmond G. Powe · Cindy M. Staka · Emad A. Rakha · Graham Ball · Andrew R. Green · Mohammed Aleskandarany · E. Claire Paish · R. Douglas Macmillan · Robert I. Nicholson · Ian O. Ellis · Julia M. W. Gee

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Abstract Transferrin receptor (CD71) is involved in the cellular uptake of iron and is expressed on cells with high proliferation. It may be implicated in promoting the growth of endocrine resistant phenotypes within ER+/luminal-like breast cancer. We used a panel of in vitro cell models of acquired resistance to tamoxifen (TAMR), Faslodex (FASR) or severe oestrogen deprivation (MCF-7X) and the ER+ luminal MCF-7 parental line to determine CD71 mRNA expression and to study transferrin (Tf) effects on in vitro tumour growth and its inhibition. Furthermore, CD71 protein expression was assessed in a well-characterized series of patients with invasive breast carcinoma using tissue microarrays. Our results demonstrated a striking elevation of CD71 in all cell models of acquired resistance. Exogenous Tf significantly promoted growth in MCF-7-X and MCF-7 cells but more so in MCF-7-X; this growth was significantly reduced by Faslodex (FAS) or a phosphoinositide-3 kinase inhibitor (LY294002). Increased CD71 expression was associated with poor NPI score, tumour proliferation, basal CKs, p53, EGFR, HER2, steroid receptor negativity and shortened breast cancer specific survival ($P < 0.001$). On multivariate analysis, CD71 was found to be an independent prognostic factor in the ER+ cohort of patients. In conclusion, therapies of current interest in breast cancer (e.g. FAS, PI3K-inhibitors) appear able to partially impact on transferrin/CD71-promoted growth, but further investigation of this important mitogenic mechanism may assist in designing new therapeutic strategies to target highly proliferative, endocrine resistant breast cancers. CD71 appears to be a candidate marker of a subgroup of ER+/luminal-like breast cancer characterised by poor outcome and resistance to tamoxifen.

Keywords Breast carcinoma · CD71 · Oestrogen receptor · Prognosis · Tamoxifen · Transferrin · Immunohistochemistry

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Introduction

The transferrin receptor (TfR, CD71) is a type II transmembrane homodimer glycoprotein (180 kDa) involved in the cellular uptake of iron via internalization of iron-loaded transferrin [1–3]. Transferrin (Tf) is therefore an essential component of cell growth and iron-requiring metabolic processes including DNA synthesis, electron transport,

mitogenic signalling pathways and in turn, proliferation, and cell survival. Consequently, rapidly growing cells require more iron for their growth than resting cells [3].

Not surprisingly, TfR is expressed at greater levels on cells with a high proliferation rate [4]. Over-expression of endogenous TfR has also been described for various cancers including those of lung [5, 6], lymph nodes [7], colon [8], and pancreas [9], reflecting increased cell proliferation. This observation can in part be attributed to the increased need for iron as a cofactor for the ribonucleotide reductase enzyme involved in DNA synthesis of rapidly dividing cells [3].

In breast cancer, TfR expression has been shown to be up to five times higher in the malignant component compared to normal tissue [10], with expression relating closely to proliferative capacity in these tumours [11]. Moreover, within endocrine responsive breast cancer cell models such as MCF-7 (representative of the ER+ luminal clinical phenotype) there is believed to be a possible association between CD71 and oestrogen receptor signalling. Studies have revealed 17β-estradiol (E2) can up-regulate CD71 expression in a dose-dependent manner, with E2 and iron showing synergistic effects in promoting proliferation [12]. However, it remains unknown if Tf/CD71 signalling is a prominent contributor to endocrine resistant breast cancer growth, and therefore if it could provide a therapeutic target specifically for this undesirable disease state.

Previously, CD71 immunostaining [11] showed elevated expression in poorly differentiated tumours, and a relationship with metastatic potential in animal mammary adenocarcinoma models [13].

The value of CD71 as a prognostic biomarker and a predictor of response to adjuvant treatment in the ER+/luminal-like breast cancer phenotype remain largely unexplored. Therefore, in this study we assessed the biological and prognostic role of CD71 in breast cancer by: (1) Determining CD71 levels of expression in the endocrine responsive MCF-7 human breast cancer cell line as well as various sub-lines representative of acquired resistance to current endocrine agents (i.e. tamoxifen, Faslodex or severe oestrogen deprivation). (2) Examining Tf effects on in vitro endocrine response and resistant breast cancer cells growth and its inhibition, by evaluating ER blockade, phosphoinositide-3 kinase (PI3K) inhibitor LY294002 or MAPK pathway inhibitor PD98059 treatment. (3) Studying the clinical relevance of CD71 protein expression in a large series of consecutive patients with invasive breast cancers using high throughput tissue microarrays (TMAs) and immunohistochemistry. In addition, we investigated if CD71 expression could be used to sub-classify ER+/luminal-like cancers and its prognostic role in a subset of tamoxifen-only treated patients.

Materials and methods

CD71 studies in endocrine responsive and resistant breast cancer models

Cell culture

The endocrine responsive MCF-7 human breast cancer cell line was routinely maintained in phenol-red containing RPMI medium with 5% fetal calf serum (FCS), examining cells in this study in log-phase under basal conditions. Further models examined in log-phase comprised MCF-7-derived sub-lines that had acquired resistance to various endocrine strategies as follows: (1) the ER+ MCF-7X model of acquired resistance to severe oestrogen deprivation (maintained in phenol-red RPMI medium supplemented with 5% heat-treated charcoal-stripped FCS [14] and (2) acquired anti-oestrogen resistant TAMR and FASR models (maintained in phenol-red-free RPMI medium containing 10^{-7} M 4-OH-tamoxifen or Faslodex (FAS) [15–17]. All basal media were supplemented with penicillin–streptomycin (100 U/ml), fungizone (2.5 μg/ml), and L-glutamine (4 mM).

PCR studies

MCF-7 and MCF-7X cells were grown in triplicate in their respective basal media to log-phase (7 days), seeding at 2.5×10^4 cells/dish. Total RNA was isolated using an RNA isolator kit (TriReagent; Sigma Chemicals), and 1 μg was reverse-transcribed as described previously [18]. Resultant cDNA samples were co-amplified for 25 cycles using specific primers for CD71 and β-actin. Individual RT-PCR was performed for the CD71 ligand Tf (32 cycles) and β-actin (25 cycles). Each primer set was optimized against multiple cycle numbers and annealing temperatures to ensure quality of the final product. Briefly, an initial denaturing step of 95°C for 2 min was followed by the stated cycle numbers comprising 94°C for 30 s, 55°C for 1 min and 72°C for 1 min. PCR products were separated on a 2% w/v agarose gel containing ethidium bromide, visualized by UV illumination, scanned, and densitometric values corrected for β-actin before statistically comparing by Student's *t*-test. All primers were designed using the online BLAST resource as follows:

CD71 (484 bp)	5′-TCTGCTATGGGACTATTGCTG-3′
	5′-CTGTTGCAGCCTTACTATACG-3′
β-actin (204 bp)	5′-GGAGCAATGATCTTGATCTT-3′
	5′-CCTTCCTGGGCATGGAGTCCT-3′
Transferrin (316 bp)	5′-CTGCACCAGGCTCTATCCTAG-3′
	5′-GTACCTAACTCTGCACAGGTG-3′

Cell growth studies

To compare the growth impact of exposure to Tf in MCF-7 and MCF-7X after 7 days treatment, cells were dispersed by trypsinisation, re-suspended in their respective basal media and seeded into 24-well plates at 4×10^4 cells/well in triplicate. After 24 h, Tf (4 μg/ml) was added, and growth examined at 7 days versus basal media control. Growth studies in MCF-7X cells were subsequently extended over a 15 day time course, examining Tf treatment (4 μg/ml) ± FAS (10^{-7} M), phosphoinositide-3 kinase inhibitor LY294002 (LY, 5 μM) or the MAPK pathway inhibitor PD98059 (PD, 25 μM). All media were replenished every 4 days and growth was measured by trypsin dispersion with coulter whole cell counting every 48 h.

CD71 expression in clinical breast cancer

We examined the expression of CD71 at the protein level using immunohistochemistry in various patient groups to assess its prognostic significance, as well as comparing staining across all the endocrine responsive and resistant in vitro breast cancer cell models.

Patients' cohort

The study population was derived from the Nottingham Tenovus primary breast carcinoma series of women aged 70 years or less, who presented with primary operable invasive breast carcinomas (with tumours of less than 5 cm diameter on clinical/pre-operative measurement, stage I and II) between 1988 and 1998. TMAs were used containing a series of 853 informative cases of unselected invasive breast carcinoma as previously reported [19].

This resource has been well characterised and has associated patient clinical and pathological data including age, histological grade [20], tumour size, lymph node status, Nottingham prognostic index (NPI) [21], vascular invasion (VI), development of recurrence, distant metastases (DM), and mitotic counts [22]. Clinical outcome data including survival time and disease-free interval (DFI) were maintained on a prospective basis. DFI was defined as the interval in months from the date of the primary surgical treatment to the first loco-regional or distant recurrence. Breast cancer specific survival (BCSS) was taken as the time in months from the date of the primary surgical treatment to the time of death from breast cancer. Median follow-up time was 150 months. In addition, protein expression data for a number of tumour-relevant biomarkers was available [19, 23, 24]. Table 1 summarizes the tumours' characteristics.

Patient management was based on tumour characteristics assessed by using the Nottingham prognostic index (NPI) and hormone receptor status as previously described [25]. This study was approved by the Nottingham Research Ethics Committee.

Tissue microarray and immunostaining of the cell pellets and clinical breast cancer samples

For paraffin-embedded pellet preparation, all endocrine responsive and resistant cell lines were seeded at 3×10^9 cells/150 mm dish and grown to log-phase (7 days)

Table 1 Tumours' characteristics

Variable	Total number
Age (years)	
<40	63
40–50	242
51–60	302
>60	246
Size	
≤1.5 cm	284
>1.5 cm	569
Lymph node stage	
1	543
2	231
3	76
Tumour grade	
1	161
2	273
3	420
Nottingham prognostic index (NPI)	
Poor	123
Moderate	492
Good	238
Distant metastasis (DM)	
No	583
Positive	262
Recurrence	
No	478
Positive	359
Vascular invasion (VI)	
No	498
Probable	85
Definite	267
Tumour type	
Ductal/NST	485
Lobular	94
Tubular and tubular mixed	188
Medullary	28
Other special types[a]	15
Mixed[b]	43

[a] Include mucoid, invasive cribriform and invasive papillary carcinoma

[b] Include ductal/NST mixed with lobular or special types

in their basal media. Cells were then removed by scraping and cell suspensions centrifuged at 1,000 rpm for 5 min. The resultant pellets were fixed in 4% formaldehyde in PBS. Pellets were combined with a 1:1 volume of 4% agar at 60°C prior to setting overnight at 4°C. Agar-cell plugs were then cut into 5 mm lengths, re-fixed for 2 h in 4% formaldehyde, dehydrated to xylene and paraffin-embedded.

Tissue microarrays from the pellets and clinical breast tissue samples were constructed using a Beecher systems microarrayer (Beecher Instruments, Inc. San Prairie, USA), at 0.6 mm and arraying 5 experimental replicates/cell lines prior to 4 μm sectioning for CD71 immunohistochemical assay. For clinical material, arrayed samples comprised single representative 0.6 mm tissue cores taken from each tumour block, sectioned at 4 μm thickness [26].

Immunohistochemical staining of TfR (CD71; clone 10F11, ab49517; Abcam, Cambridge, UK) was performed as previously described [27]. Signal localization (plasma membrane, cytoplasmic) and the staining intensity was quantified using *H*-score (histochemical score) analysis considering the invasive tumour component only [28]. Standard cut-off values needed to determine categorical scores before statistical analysis were the same as those published in previous studies [19, 23, 24]. HER2 was scored according to the Herceptest scoring guidelines (DakoCytomation, Cambridge, UK).

Statistical analysis

Statistical analysis was performed using SPSS 15.0 statistical software (SPSS Inc., Chicago, USA). Cell line growth data obtained were log-transformed to compare growth rate at day 15, using ANOVA followed by a Bonferroni post hoc test for analysis. *H*-scores were compared statistically between MCF-7 and the resistant models using Student's *t*-test with post hoc testing. Association between the CD71 expression (categorised by the median of the *H*-score ≥ 5) and different clinicopathological parameters and biomarkers was evaluated using chi-square test. Survival curves were estimated by the Kaplan–Meier method with a log rank test to assess significance. Multivariate cox regression analysis was used to evaluate any independent prognostic effect of the variables using 95% confidence interval. A *P* value of <0.05 was considered as significant.

Results

Endocrine responsive and resistant breast cancer cell line studies

Immunocytochemistry on the cell pellets revealed CD71 expression was increased in all cell lines derived from the luminal ER+ MCF-7 model that had undergone progression to endocrine resistance (Fig. 1i), with increased immunoreactivity localised at the plasma membrane and cytoplasm. Of these models, total CD71 expression was most elevated for the MCF-7X line with ~ 5 fold increase versus MCF-7 (mean *H*-scores = 144 vs 28.5).

RT-PCR confirmed that MCF-7X cells had substantially increased CD71 mRNA expression versus parental MCF-7 cells ($P < 0.001$), but the production of endogenous Tf ligand was very low and at an equivalent level in both models (Fig. 2a). Tf significantly stimulated MCF-7 ($P < 0.001$) and particularly MCF-7X cell growth ($P < 0.001$) versus their respective basal control growth (Fig. 2b). Subsequent growth curve analysis of MCF-7X cells over a 15 day time course revealed Tf-induced growth could be partially depleted (by approximately 50–60%) either by treating with 5 μM phosphoinositide-3 kinase inhibitor LY294002 (60% growth decline by day 15, $P = 0.002$; Fig. 2c) or by subjecting the cells to further ER blockade using 10^{-7} M FAS (50% growth decline by day 15, $P = 0.012$; Fig. 2d). In contrast, Tf-induced growth could not be significantly decreased by the MAPK pathway inhibitor PD98059 (25 μM) in MCF-7X cells (not illustrated).

CD71 immunohistochemical results in clinical breast cancer

Correlation between CD71 expression and clinicopathological variables

The observed CD71 staining pattern in tumour tissues was both plasma membranous and cytoplasmic (Fig. 1ii).

The level and extent of staining varied from very weak focal to extensive strong overexpression (*H*-score range = 5–300). CD71 overexpression was associated with larger tumour size, higher histologic grade and poorer NPI group and distant metastases. It was associated with the proliferative activity of tumours as assessed by mitotic counts and MIB-1 expression ($P < 0.001$). CD71 expression was positively associated with other markers of aggressive tumour phenotype including basal CKs (CK14 and CK5/6), p53, EGFR, and HER2. In contrast, CD71 expression was inversely related to ER, progesterone receptor (PgR), androgen receptor (AR) and Bcl-2 expression. We found higher levels of expression of CD71 in medullary type cancer compared with others (89%, $P < 0.001$; Tables 2, 3).

In the ER+/luminal-like tumours ($n = 533$), CD71 expression showed a positive association with higher grade ($P < 0.001$) and poorer NPI ($P = 0.004$), distant metastasis ($P = 0.002$), high mitotic counts ($P < 0.001$), and increased MIB-1 expression ($P = 0.030$). CD71 expression

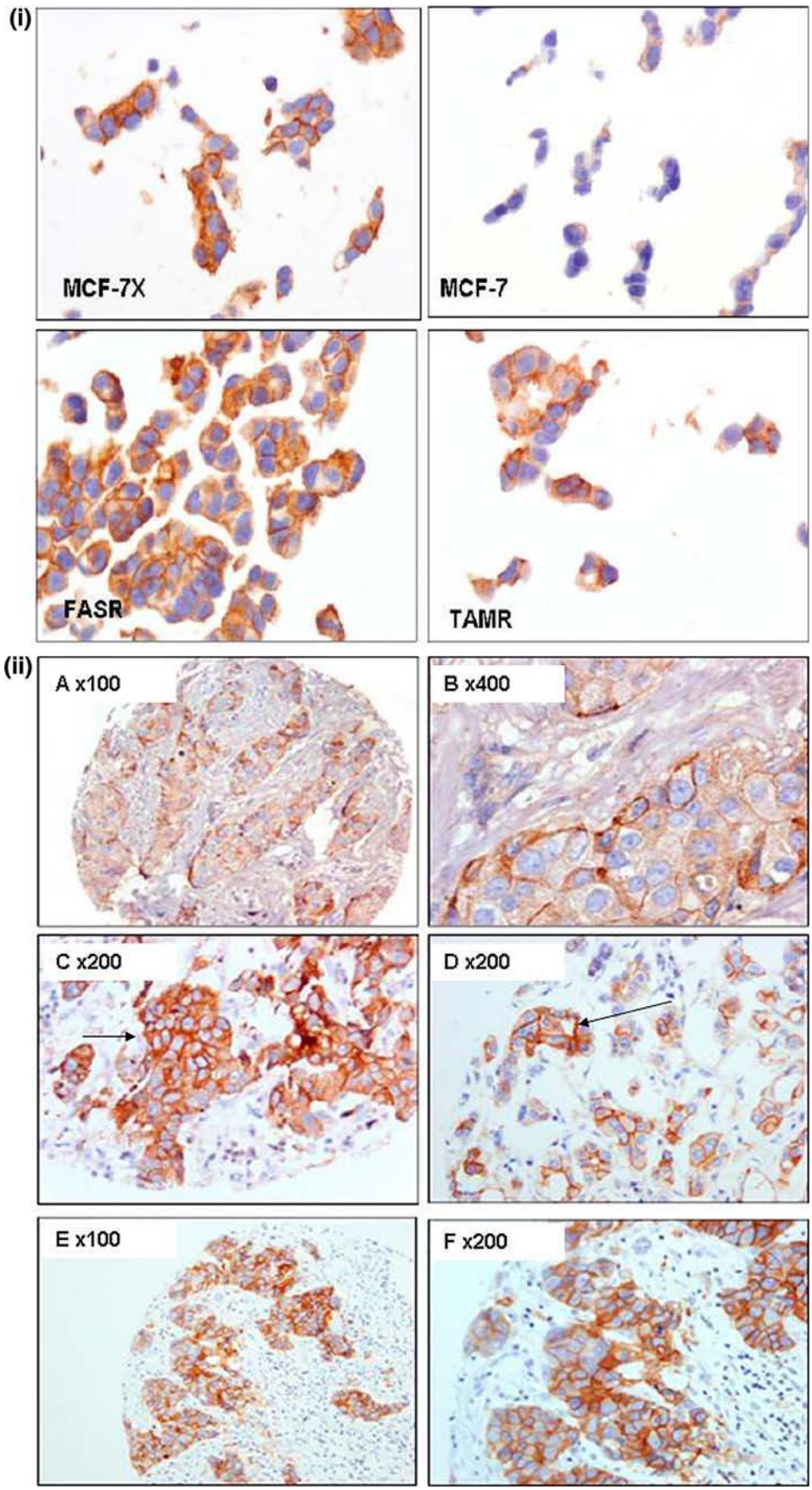

Fig. 1 CD71 immunohistochemistry **i** Paraffin-embedded cell pellets (×400): total CD71 expression was most elevated in the MCF-7X. **ii** Clinical breast cancer cases: (*A* and *B*) grade III ductal carcinoma with positive membranous and cytoplasmic staining (*arrow*). (*C*) High grade ductal carcinoma with positive membranous and cytoplasmic staining (*D*) grade II ductal carcinoma with positive membranous staining (*arrow*). (*E* and *F*) Grade III ductal carcinoma with positive membranous staining

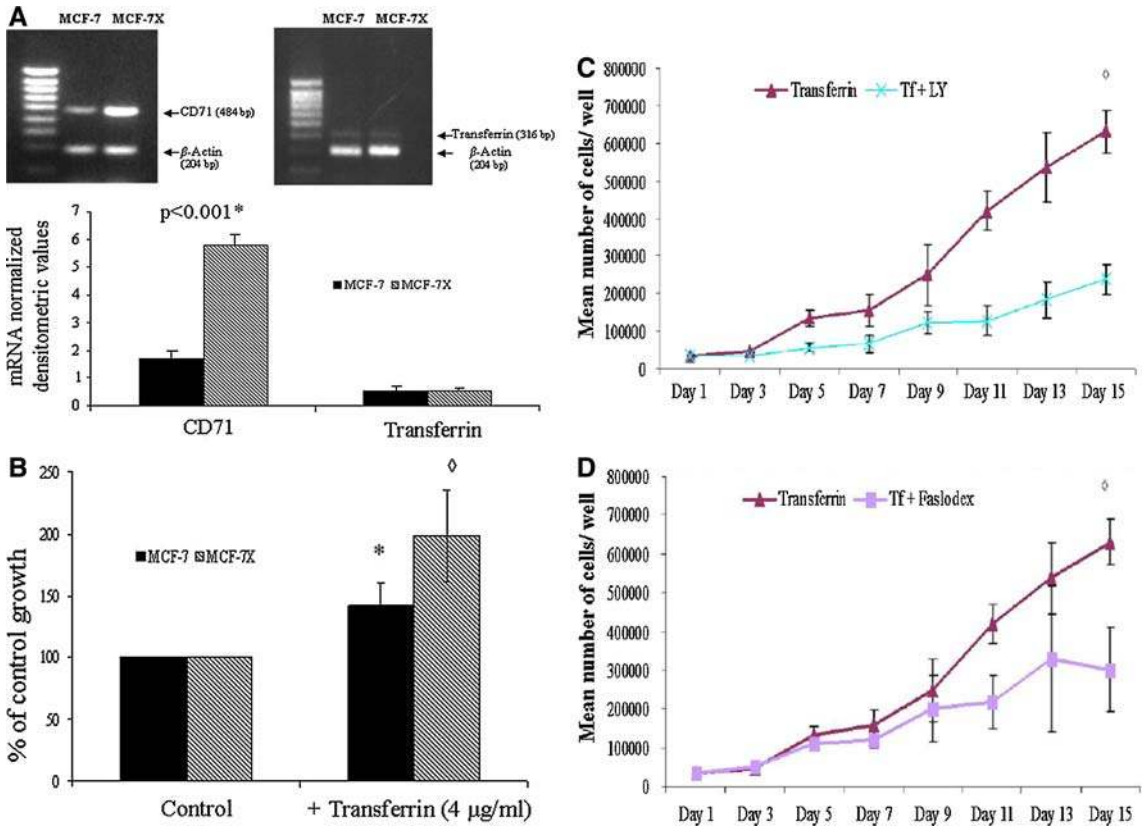

Fig. 2 **a** mRNA expression of CD71 and transferrin in MCF-7 versus MCF-7X cells at 7 days as measured by RT-PCR. Digital images are representative of 3 experiments and the statistical analysis applied was Student's *t*-test comparing mean MCF-7 cell and MCF-7X cell expression normalised to β-actin. **b** Effect of transferrin (4 µg/ml) on growth of MCF-7 versus MCF-7X cells at 7 days. Data are displayed as a percentage of control cell growth ($n = 3 \pm$ SD). Transferrin significantly stimulated * MCF-7 ($P < 0.001$) and ◇ MCF-7X cell growth ($P < 0.001$) versus their respective basal control growth. **c** Growth challenge with transferrin (4 µg/ml) ± LY294002 (5 µg/ml) in MCF-7X cells over 15 days time course. The data are displayed as the mean number of cells/well ± SD ($n = 3$). ◇ denotes transferrin + LY294002-treated growth was significantly different ($P = 0.002$) versus transferrin alone at day 15 applying ANOVA with Bonferroni post hoc test. **d** Growth challenge with transferrin (4 µg/ml) ± FAS (10^{-7} M) in MCF-7X cells over 15 days time course. The data is displayed as the mean number of cells/well ± SD ($n = 3$). ◇ denotes transferrin + FAS-treated growth was significantly different ($P = 0.012$) versus transferrin alone at day 15 applying ANOVA with Bonferroni post hoc test

was positively associated with p53 ($P < 0.001$), EGFR ($P < 0.001$), CK5/6 ($P = 0.029$), HER2 overexpression ($P = 0.010$), and negative expression of AR ($P = 0.038$).

Correlation between CD71 protein expression and patient outcome

In the whole series, a significant correlation between CD71 expression and poorer BCSS was identified [Log Rank (LR) = 14.833, $P < 0.001$]. In the ER+/luminal-like cohort, we also found a significant association (LR = 14.044, $P < 0.001$; Fig. 3a). However, no associations were found between CD71 expression and DFI neither in the whole series (LR = 3.132, $P = 0.077$) nor in the ER+ patient group (LR = 2.121, $P = 0.145$). In the ER negative group, CD71 expression was not related to BCSS or DFI.

In the group of ER+ Tf only treated patients ($n = 180$), we found that CD71 expression was associated with shorter BCSS (LR = 10.345, $P = 0.001$; Fig. 3b) and shorter DFI (LR = 4.056, $P = 0.044$; Fig. 3c) which may indicate poor response of CD71 expressing tumours to hormonal treatment.

To futher explore this finding, we examined outcome in ER+ CD71 + patients that received or did not receive tamoxifen, the group of patients who received tamoxifen showed a significant lower BCSS (LR = 5.571, $P = 0.018$; Fig. 3d) and shorter time to develop distant metastasis (LR = 5.360, $P = 0.021$), suggesting adverse imapct of tamoxifen in these CD71+ ER+ patients.

Multivariate analysis

A multivariate Cox hazard model analysis for predictors of BCSS was performed including CD71 expression, tumour size, histologic grade and lymph node stage. This analysis demonstrated that CD71 expression is an

Table 2 Relation of CD71 protein expression to the other clinicopathological parameters in the whole series

Variable	Negative CD71 N (%)	Positive CD71 N (%)	Total	χ^2	*P* value	Correlation coefficient
Age						
<40	23 (36.5)	40 (63.5)	63	0.634	0.888	-0.011
40–50	78 (32.2)	164 (65.6)	242			
51–60	104 (35)	198 (65)	302			
>60	86 (34.1)	160 (65.9)	246			
Size						
≤1.5 cm	115 (40.5)	169 (59.5)	284	7.706	**0.006**	0.090
>1.5 cm	176 (30.9)	393 (69.1)	569			
LN Stage						
1 (negative)	192 (35.5)	351 (64.5)	543	1.238	0.539	0.036
2 (1–3 LN)	73 (31.5)	158 (68.5)	231			
3 (>3 LN)	24 (31.6)	52 (68.4)	76			
Grade						
1	81 (50.3)	80 (49.7)	161	78.847	**<0.001**	0.280
2	128 (47.1)	144 (52.9)	273			
3	82 (19.5)	338 (80.5)	420			
NPI						
Poor	28 (23)	95 (77)	123	38.912	**<0.001**	0.197
Moderate	144 (29)	348 (71)	492			
Good	119 (50)	119 (50)	238			
DM						
No	220 (37.7)	363 (62.3)	583	10.439	**0.001**	0.111
Positive	69 (26.3)	193 (73.7)	262			
Recurrence						
No	172 (36)	306 (63)	478	1.855	0.173	0.047
Positive	113 (31.5)	246 (68.5)	359			
VI						
No	179 (35.9)	319 (64.1)	498	1.787	0.409	0.043
Probable	27 (31.8)	58 (68.2)	85			
Definite	84 (31.5)	183 (68.5)	267			
Mitotic count						
1	153 (55.6)	122 (44.4)	275	98.89	**<0.001**	0.330
2	52 (35.1)	96 (64.9)	148			
3	72 (18.5)	317 (81.5)	389			
Tumour type						
Ductal/NST	121 (24.9)	364 (75.1)	485	65.803	**<0.001**	
Lobular	56 (60)	38 (40)	94			
Tubular and tubular mixed	84 (44.7)	104 (55.3)	188			
Medullary	3 (10.7)	25 (89.3)	28			
Other special types[a]	8 (53.3)	7 (46.7)	15			
Mixed[b]	19 (44.2)	24 (55.8)	43			

Note: Significant *P* values are indicated in bold

[a] Include mucoid, invasive cribriform and invasive papillary carcinoma

[b] Include ductal/NST mixed with lobular or special types

independent prognostic factor in the ER+/luminal-like patient group (HR = 1.614, 95% CI = 1.092–2.384 and $P = 0.016$).

Importantly, in ER+ patient who received tamoxifen only, CD71 was shown to be an independent prognostic factor of BCSS (HR = 2.624, 95% CI = 1.309–5.259 and

Table 3 Relation of the CD71 protein expression to other biomarkers in the whole series

Variable	CD71 expression		Total	χ^2	*P* value	Correlation coefficient
	Negative CD71 N (%)	Positive CD71 N (%)				
CK5/6						
Negative	247 (37.8)	407 (62.2)	654	20.527	**<0.001**	0.158
Positive	32 (19.2)	135 (80.8)	167			
CK14						
Negative	239 (35.8)	428 (64.2)	667	10.694	**0.001**	0.115
Positive	29 (21.3)	107 (78.7)	136			
CK18						
Negative	142 (33.8)	278 (66.2)	420	0.280	0.579	−0.020
Positive	111 (35.5)	200 (64.5)	311			
HER2						
Negative	259 (37.2)	437 (62.8)	696	23.084	**<0.001**	0.169
Positive	15 (13.8)	94 (86.2)	109			
P53						
Negative	241 (41.9)	334 (58.1)	575	51.014	**<0.001**	0.254
Positive	32 (14.8)	184 (85.2)	216			
EGFR						
Negative	191 (37.2)	323 (62.8)	514	16.316	**<0.001**	0.157
Positive	29 (19.5)	120 (80.5)	149			
ER						
Negative	50 (18.7)	217 (81.3)	267	46.012	**<0.001**	−0.240
Positive	229 (43)	304 (57)	533			
AR						
Negative	78 (25.6)	227 (74.4)	305	18.196	**<0.001**	−0.156
Positive	179 (40.7)	261 (59.3)	440			
PgR						
Negative	90 (25.6)	261 (74.4)	351	24.011	**<0.001**	−0.174
Positive	186 (42.4)	253 (57.6)	439			
MIB1						
Low	75 (42.1)	107 (58.8)	182	12.289	**<0.001**	0.172
High	58 (25)	174 (75)	232			
Bcl2						
Negative	29 (21.8)	104 (78.2)	133	16.373	**0.001**	−0.152
Weak	37 (36.6)	64 (63.4)	101			
Moderate	69 (44.2)	87 (55.8)	156			
Strong	21 (38.2)	34 (61.8)	55			

Note: Significant *P* values are indicated in bold

$P = 0.007$; Table 4), where patients with CD71 positive tumours showed shorter BCSS.

Discussion

In recent years, increasing effort has focused on classifying breast cancer using different approaches, including gene expression arrays and TMAs [19, 29]. Gene expression studies have been able to classify breast cancer into a number of different distinct biological classes which have relationships with clinical outcome [29–34]. One such class is the luminal group which is characterised by high ER expression and expression of luminal epithelial cell characteristics [35]. Luminal A has higher expression of ER-related genes and lower expression of proliferative genes than the luminal B subtype, while luminal C cancers were reported to have some features that were similar to those

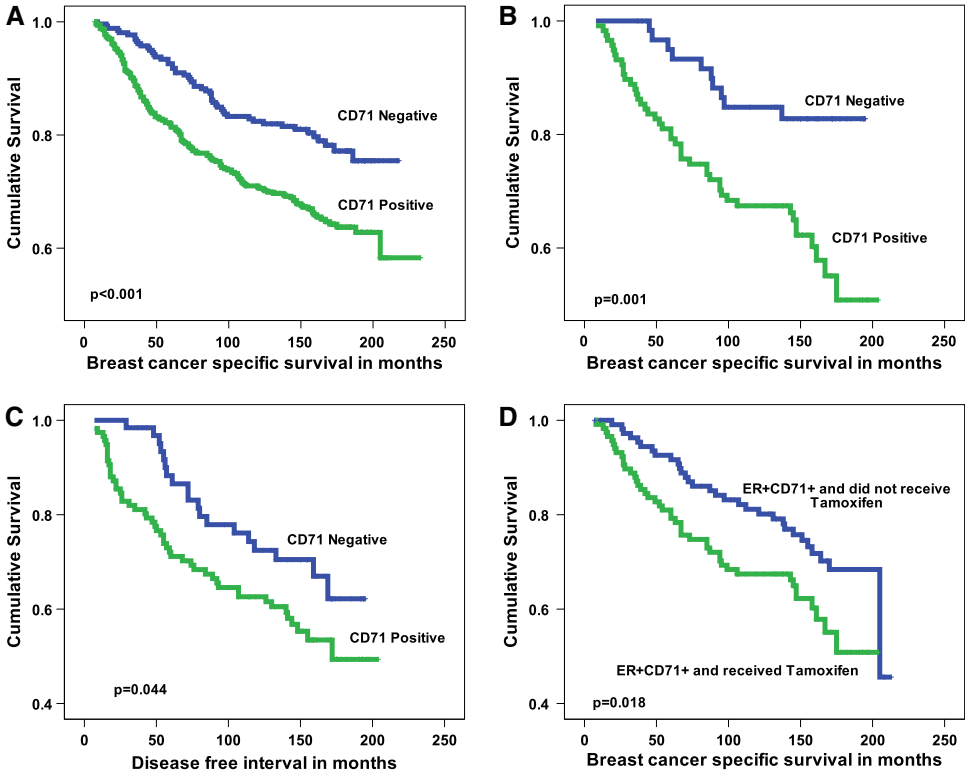

Fig. 3 Kaplan Meier plots of CD71 expression and BCSS in **a** ER+ cohort of unselected breast cancer patients, **b** ER+ tamoxifen only treated patients. **c** Kaplan Meier plot of CD71 expression and DFI in ER+ tamoxifen only treated patients. **d** Kaplan Meier plot of BCSS of patients who received or did not receive tamoxifen in CD71+ ER+ cohort

Table 4 Cox proportional hazards analysis for predictors of BCSS: effects of tumour grade, size, lymph node stage, and CD71 expression in (A) ER+ cohort and (B) ER+ tamoxifen only treated patients

Variable	Hazard ratio	95% CI	*P* value
(A) ER+ cohort			
Tumour grade	2.182	1.659–2.871	**<0.001**
Tumour size ≥1.5 cm	1.803	1.167–2.786	**0.008**
Lymph node status	1.911	1.335–2.735	**0.001**
CD71 expression	1.614	1.092–2.384	**0.016**
(B) ER+ tamoxifen only treated patients			
Tumour grade	2.470	1.329–3.261	**0.001**
Tumour size ≥1.5 cm	1.335	0.909–2.703	0.393
Lymph node status	2.034	1.484–2.592	**0.019**
CD71 expression	2.624	1.309–5.259	**0.007**

Note: Significant *P* values are indicated in bold

found in the Sorlie's basal group [32, 35]. Luminal B/C tumours have a poorer outlook, possibly resulting from their increased proliferation rate [33]. However, the position regarding the number of classes within the luminal group remains controversial [30, 35] and this has prompted the need to identify candidate biomarkers to better subclassify them with respect to patient outcome and response to different treatment strategies. Tf acting via CD71 has been shown to alter during disease progression and may promote aggressive tumour growth [36]. Because of these various associations and presence of CD71 gene expression in luminal group C by Sorlie et al. [35], we propose that assessment of CD71 expression might equally be used to stratify ER+ patients to define subgroups with poor prognosis, high proliferation and resistance to hormonal therapy. The present study has shown for the first time that elevated CD71 is a feature of endocrine resistant breast cancer, as evidenced by immunostaining of acquired endocrine resistant sub-lines derived from luminal-like MCF-7 cells. Furthermore, we showed using the model for resistance to severe oestrogen deprivation, MCF-7X, that endocrine resistant cells overexpress CD71 at the gene and protein level and that exogenous Tf leads to their increased growth. This could thus represent a prominent mitogenic mechanism for endocrine resistant cells in the presence of circulating Tf.

Studies in breast cancer models are promising with antisense inhibition of CD71 or selective antibodies to this receptor, where these inhibit cell survival and proliferation confirming a fundamental growth importance of CD71 to such cells [37]. Peng et al. [38] suggested the use of intracellular antibody technology targeted against CD71 in CD71-overexpressing cancer. The use of monoclonal antibodies against TfR and ascorbate to inhibit both cell proliferation and the pro-angiogenic hypoxia inducible factor HIF-1α may also be of therapeutic use [39]. Tf/CD71

trafficking has also been closely associated with PI3K where inhibitors of this intracellular kinase appear able to deplete cell surface CD71 level [40]. Our finding that FAS and the phosphoinositide-3 kinase inhibitor (LY294002) can partially deplete Tf-induced growth of MCF-7X cells implies mechanistic cross-talk between Tf/CD71 mitogenic signalling, ER and PI3K (in contrast to an apparent lack of CD71 interplay with MAP kinase) in ER+ endocrine resistant cells, suggesting further therapeutic approaches.

Our retrospective tissue studies support the concept that there is a need for increased iron uptake mediated through elevated CD71 protein levels in high grade breast tumours, characterised by poor NPI, large size and, as predicted, high mitotic activity. Consequently, CD71 expression was more frequently increased in medullary carcinoma and basal-like tumours (CK5/6+ and CK14+) that show these features [41–43]. Furthermore, CD71 expression was also significantly associated with other markers of aggressive phenotype and endocrine treatment resistance including p53, HER2 and EGFR [44].

Tumours with elevated CD71 expression had a shorter BCSS in the whole patient series and in the ER+/luminal-like patient cohort. These results confirmed that CD71 expression can define poor clinical outcome in the ER+ patient group. Supporting this, CD71 expression was found to be an independent prognostic marker in the ER+ cohort. In considering ER+ tamoxifen-only treated patients, increased CD71 expression was associated with shorter BCSS and DFI suggestive that there might (as in vitro) be a relationship between CD71 expression and adverse endocrine response.

In conclusion, the present study demonstrates that prominent expression of CD71 protein is a feature of breast cancers with poor prognosis and as such, we propose that TfR expression may have implications for diagnosis and prognosis. CD71 protein expression could be of value in characterizing a subset of ER+/luminal-like tumours with poor prognosis in clinical practice, as well as defining patients less likely to respond to endocrine therapy. Therapies of current interest in breast cancer (e.g. FAS, PI3K-inhibitors) appear able to partially impact on Tf/CD71-promoted growth, but further investigation of this important mitogenic mechanism may assist in designing new therapeutic strategies to target highly proliferative, endocrine resistant breast cancers. Therapies targeting iron delivery or CD71 itself, may have therapeutic benefits in treating CD71+ ER+ breast cancers in the clinic.

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