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PRECLINICAL STUDY

Nuclear and cytoplasmic expressions of ERβ1 and ERβ2 are predictive of response to therapy and alters prognosis in familial breast cancers

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Abstract Estrogen receptor (ER) α has been studied extensively in familial breast cancers but there are limited data on ERβ and its isoforms. This is an important issue since many BRCA1-associated tumours are "triple negative" and are resistant to conventional and targeted therapies. We performed an immunohistochemical study of pan-ERβ, ERβ1 and ERβ2 in a cohort of 123 familial breast carcinomas (35 BRCA1, 33 BRCA2 and 55 BRCAX) using a cut-off for positivity at 20% (Shaaban et al. in Clin Cancer Res 14:5228–5235, 2008). BRCA1 cancers were more likely to be nuclear ERα negative and nuclear pan-ERβ positive (21/32, 66%) when compared with BRCA2 (2/29, 7%) and BRCAX cancers (11/49, 22%) (both $P < 0.001$). For survival analysis, expression was also stratified using cut-offs defined by Bates et al. (Breast Cancer Res Treat 111:453–459, 2008) (score out of 7). Cytoplasmic ERβ2 expression correlated with shorter overall survival at 15 years regardless of cut-off used (both $P < 0.046$) At a cut-off score of 6 out of 7, cytoplasmic ERβ2 expression correlated with a poorer response to chemotherapy in both univariate ($P = 0.011$) and multivariate analyses including grade, lymph node status and chemotherapy as an interaction variable ($P = 0.045$, Hazard ratio 1.22, 95% CI 1.004–9.87). A similar trend was seen in a univariate analysis with a cut-off of 20% although this did not reach statistical significance ($P = 0.057$). Expression of nuclear ERβ1 was associated with a favourable response to endocrine therapy at 15 years regardless of cut-offs employed (both $P < 0.025$). However, this did not reach statistical significance in a multivariate analysis ($P > 0.05$). Since a significant proportion of ERα negative familial breast carcinomas are positive for nuclear ERβ1 and cytoplasmic ERβ2, the different ERβ isoforms and their intracellular location may need to be assessed, to identify patients that may benefit from hormonal and chemotherapy.

Keywords ERβ · Breast · BRCA1 · BRCA2 · BRCAX · Familial breast carcinoma

Introduction

It is estimated that 5–10% of all breast cancers are attributable to inherited mutations in susceptibility genes, of which the two most important are BRCA1 and BRCA2 [1]. BRCA1 tumours show a so called triple negative phenotype being oestrogen receptor (ER), progesterone receptor (PgR) and HER2 negative [1]. They also harbour p53 mutations [2] and express basal and myoepithelial markers [3–5]. No similarly defined phenotype has been described for BRCA2 tumours which usually show a ductal, no special type morphology and ER positivity [6].

Strategies using selective oestrogen receptor modulators such as tamoxifen-targeting tumours that express ERα have resulted in improvements in relapse-free and overall survival [7]. The advent of trastuzumab and lapatinib has similarly revolutionized treatment for HER2-amplified breast cancer [8, 9]. However, the resistance of BRCA1

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basal-like breast cancers to conventional agents have made these tumours difficult to treat [10]. Nevertheless, there are data to suggest that tamoxifen may prevent development of contralateral breast cancer in women with a strong family history and increases survival in BRCA1 breast-cancer-affected patients [11, 12].

Estrogens mediate their role in the progression of breast cancer and through two transcription factors, ERα and ERβ. It is becoming apparent that ERβ plays an important role in breast cancer progression [13]. In contrast to ERα, ERβ expression is progressively lost during transition from normal breast to invasive carcinoma [14, 15]. Recent studies on sporadic breast cancers have shown that the expression of ERβ isoforms 1, 2 and 5 may have important prognostic implications [16–18].

To our knowledge, there are two reports documenting the expression of ERβ in familial breast cancers. The first study in 44 familial cancer patients (16 BRCA1, 12 BRCA2 and 16 BRCAX) [19] and the second in 48 patients with BRCA1 founder mutation positive women [20]. However, there are no data regarding the range of expression for its different isoforms and the cellular pattern of expression for either BRCA1- or BRCA2-associated tumours. Thus, we have performed a comprehensive immunohistochemical analysis of pan-ERβ, ERβ1 and ERβ2 in a large cohort of BRCA1, BRCA2 and BRCAX tumours with survival data. Our aims are to document the range of expression of ERβ in familial breast cancer, and to determine the relationship between ERβ and conventional clinicopathological parameters and survival. We have also evaluated the relationship between ERβ and intrinsic breast cancer subtypes including basal-like, luminal, HER2 and null types.

Materials and methods

Patients

147 cases of familial breast carcinomas from female patients were collected from the kConFab biorepository between 1980 and 2005. Classification of BRCA1 and BRCA2 mutations and sequence variants was according to designations listed for research purposes on the kConFab website (www.kconfab.org). The BRCAX breast cancers are defined by breast cancer in families without a known BRCA1 and BRCA2 pathogenic mutation, who met kConFab category 1 and 1B eligibility criteria. Of the 147 cases, 18 cases were excluded due to the lack of tissue available and a further 6 cases were excluded due to the absence of tumour on the array. The final cohort was composed of 123 cases including 35 BRCA1, 33 BRCA2 and 55 BRCAX cases (Table 1). All the patients had operable breast carcinomas and were not diagnosed with metastatic disease at the time of presentation. Patients were followed up for a median period of 64.0 (range 0.4–298.8) months. During this time, 38 patients relapsed and 31 died from breast cancer (deaths unrelated to breast cancer were censored). Breast-cancer-specific survival was defined as time from primary surgical excision to breast-cancer-related death.

Using stratification of intrinsic phenotypes based on Nielsen et al. [21] tumours were placed into luminal (ERα positive, HER2 negative, cytokeratin (CK) 5/6 negative or positive), basal (HER2 and ERα negative; CK5/6 positive), HER2 (HER2 positive, ERα and CK5/6 negative or positive) and null/negative (HER2, ERα and CK5/6 negative).

Immunohistochemistry

Tumour tissue microarrays (1-mm cores), with a fourfold redundancy, were prepared from formalin-fixed, paraffin-embedded tissue blocks of 129 tumours. Sections were cut, dewaxed, placed through graded alcohol and water. Antigen retrieval was performed in PT Link using low pH EnVision FLEX Target Retrieval Solution (Dako, Denmark) for 20 min at 100°C. Endogenous peroxidase was blocked with EnVision FLEX Peroxidase-Blocking Reagent (Dako, Denmark) before incubating the sections with mouse monoclonal antibodies pan-ERβ (Clone 14C8, Abcam, 1/200), ERβ1 (Clone PPG5/10, GeneTex, 1/15) [17] and ERβ2 (Clone 57/3, Serotec, 1/10) [18] for overnight at 4°C. Antigen–antibody complex was detected using Envision FLEX system (EnVision FLEX/HRP and EnVision FLEX DAB+ Chromogen). The specificity of both nuclear and cytoplasmic staining for these antibody clones have been previously established via peptide absorption studies [18, 22].

HER2 chromogenic in situ hybridisation (CISH) and immunoperoxidase staining for ERα, PgR, HER2, CK5/6 and EGFR were performed for all tumours. HER2 CISH was performed using the Invitrogen Spotlight system (Invitrogen, California, USA). Tumour cells were regarded as positive for amplification if there were more than six signals per nucleus [23]. Tumours in the equivocal group (4–6 signals) were further probed with chromosome 17 and considered amplified with a ratio of >2.2 [24].

Immunohistochemical scoring and cut-off levels

ERβ was scored for nuclear and cytoplasmic staining using a cut-off of 20%, as defined by Shaaban et al. [18]. For survival analysis, the data were also analysed using cut-offs defined by Bates et al. [25]: the intensity of staining was scored as negative = 0, weak = 1, moderate = 2 or strong = 3 (Figure S1). The percentage of tumour cells was scored as: 0 = 0; 1–10 = 1, 11–50 = 2, 51–80 = 3,

Table 1 Clinical and tumour characteristics (n = 123)

	BRCA1, *n* (%)	BRCA2, *n* (%)	BRCAX, *n* (%)	All familial, *n* (%)
Age				
Median (range), years				
<40 years	13 (37)	9 (27%)	9 (16%)	31 (25%)
40–55 years	20 (57)	13 (38%)	29 (53%)	62 (50%)
55–69 years	2 (6)	9 (27%)	14 (25%)	25 (20%)
>70 years	0	2 (6%)	3 (6%)	5 (4%)
Tumour size				
<20 mm	26 (74%)	16 (52%)	26 (54%)	68 (60%)
>20 mm	9 (26%)	15 (48%)	22 (46%)	46 (40%)
Unknown	0	2	7	9
Nodal status				
Negative	32 (91%)	23 (74%)	31 (65%)	86 (76%)
Positive	3 (9%)	8 (26%)	17 (35%)	28 (24%)
Unknown	0	2	7	9
Grade				
I	0	1 (4%)	6 (13%)	7 (7%)
II	2 (7%)	14 (48%)	14 (30%)	30 (29%)
III	27 (93%)	14 (48%)	26 (57%)	67 (64%)
Unknown	6	4	9	19
ER alpha				
Negative	27 (84%)	5 (17%)	16 (33%)	48 (44%)
Positive	5 (16%)	24 (83%)	33 (67%)	62 (56%)
Unknown	3	4	6	13
PgR				
Negative	27 (84%)	10 (35%)	23 (47%)	60 (55%)
Positive	5 (16%)	19 (65%)	26 (53%)	50 (45%)
Unknown	3	4	6	13
HER2 status				
Negative	27 (100%)	25 (100%)	39 (87%)	91 (94%)
Positive	0	0	6 (13%)	6 (6%)
Unknown	8	8	10	26
Endocrine therapy				
Not given	26 (90%)	19 (70%)	28 (60%)	65 (71%)
Given	3 (10%)	8 (30%)	19 (40%)	38 (29%)
Unknown	6	6	8	20
Chemotherapy				
Not given	10 (35%)	15 (56%)	21 (45%)	46 (45%)
Given	19 (65%)	12 (44%)	26 (55%)	57 (55%)
Unknown	6	6	8	20

81–100 = 4. The intensity and the percentage of positive tumour cells were added together to give a maximum score of 7. Previously defined median cut-offs of 7 for nuclear expression and 6 for cytoplasmic expression were used to groups into positive and negative tumours [25]. The highest score from the 4 cores of the tissue array was used where any discordance between cores was noted. For HER2, EGFR and CK5/6, the same cut-offs were derived from Neilsen et al. An Allred score of >2/8 was considered as positive for ERα [26].

Statistical analysis

Correlations were evaluated using, the Kruskal–Wallis or Chi-square tests where appropriate. Kaplan–Meier survival curves were calculated for breast-cancer-specific death, and were compared at 5 and 15 years using a log rank test. Binary logistic regression was used for multivariate analyses and the Cox proportional hazard regression model was used to identify independent prognostic factors for breast-cancer-specific survival. Analyses were performed

with SPSS 16.0 (SPSS Inc., IL, USA). A 2-tailed P value test was used in all analyses and a P value of less than 0.05 was considered as statistically significant.

Results

ERβ expression in familial breast cancers and their relationship with clinicopathological parameters

Pan-ERβ was expressed both in the nuclear and in the cytoplasmic compartments. This ranged from focal weak positivity to widespread strong positivity. Using a cut-off of 20% staining the most common nuclear ER phenotype in BRCA1 cancers was n(nuclear)ERα-n(pan)ERβ+ (65.6%) (Table 2). BRCA1 cancers were significantly more likely have nERα–nERβ− and nERα–nERβ+ phenotype when compared with BRCA2 and BRCAX cancers (both $P < 0.001$). In contrast, BRCA2 and BRCAX cancers were significantly ($P < 0.001$) more likely to be nuclear ERα positive (the most common phenotype being nERα+ nERβ+) (75.9 and 61.2%, respectively) when compared with BRCA1 (12.5%) (Table 2).

The distributions of pan-ERβ, ERβ1 and ERβ2 expressions stratified by BRCA status and intrinsic phenotypes are shown in Tables S1a–S1f. Correlation with ERβ expression was performed using a cut-off of 20% [18]. No differences in pan-ERβ, ERβ1 or ERβ2 expression when tumours were stratified by BRCA status (all $P > 0.05$). Basal-type familial breast cancers were significantly less likely to be nuclear pan-ERβ positive (26/36, 72.2%) when compared with luminal familial cancers (44/49, 89.8%) ($P = 0.036$). There were no significant differences in ERβ1 and ERβ2 expressions between familial luminal and basal cancers ($P > 0.005$).

ERα expression correlated with positive nuclear pan-ERβ expression ($P = 0.014$) and negative cytoplasmic ERβ2 expression ($P = 0.024$). Patients with positive cytoplasmic ERβ2 were more likely to receive chemotherapy ($P = 0.042$) (Table 3). There was no significant association between pan-ERβ, ERβ1 or ERβ2 expression and tumour size, grade, lymph node status, PR, Her-2 and treatment with endocrine therapy (all $P > 0.05$).

ERβ and survival analysis in familial breast cancers

Analyses for survival were performed at 5 and 15 years using two different cut-offs as defined by Shaaban et al. (20% staining) and Bates et al. (7 out of 7 nuclear staining and 6 out of 7 cytoplasmic staining). Expression of cytoplasmic ERβ2 in familial breast cancers correlated with shorter breast-cancer-specific survival at 15 years irrespective of cut-offs employed ($P = 0.045$ at 20% and $P = 0.002$ at score of 6) (Table 4; Fig. 1a, b). A similar trend was seen at 5 years although this was not statistically significant ($P = 0.226$ at 20% and $P = 0.061$ at score of 6). There was a trend for nuclear ERβ1 expression to be associated with survival at 15 years although this was not statistically significant ($P = 0.229$ at 20% and $P = 0.064$ at score of 7). There was no correlation between survival and pan-ERβ, cytoplasmic ERβ1 or nuclear ERβ2 expression ($P > 0.05$).

The tumours were then analysed based on their intrinsic subtypes. For basal-type familial breast cancers, cytoplasmic ERβ2 expression was associated with shorter overall survival at 5 and 15 years at a cut-off of 6 out of 7 ($P = 0.039$ and $P = 0.011$, respectively) (Table 4; Fig. 1d). At a cut-off of 20% a similar trend was seen although it did not reach statistical significance ($P = 0.182$ at 5 years and $P = 0.159$ at 15 years) (Table 4; Fig. 1c). No differences in survival were observed for luminal, HER2 and null subtypes when stratified by nuclear or cytoplasmic ERβ expression (all $P > 0.05$).

When the tumours were analysed according to their BRCA status, there was a non-significant trend for BRCA1 tumours with positive cytoplasmic ERβ2 to have a poorer 15-year survival ($P = 0.306$ at 20% and $P = 0.068$ at 6 out of 7) (Table 4; Fig. 1e, f). There was no correlation between ERβ expression and survival for BRCA2 and BRCAX cancers.

Survival analysis by treatment with endocrine therapy

Analysis of the familial cancers by treatment group was then performed. In patients treated with tamoxifen, positive nuclear ERβ1 expression correlated with longer 15-year survival ($P = 0.024$ at 20% and $P = 0.021$ at score of 7) (Fig. 2a, b). This did not reach statistical significance in a multivariate analysis using the Cox regression model, with

Table 2 Nuclear ERα and nuclear pan-ERβ phenotypes in BRCA tumors, cut-off for positive expression at 20% of tumour cells

	ERα+ ERβ− (%)	ERα+ ERβ+ (%)	ERα− ERβ−(%)	ERα− ERβ+ (%)	Total (%)
BRCA1	1 (3.1)	4 (12.5)	6 (18.8)	21 (65.6)	32 (100.0)
BRCA2	3 (10.3)	22 (75.9)	2 (6.9)	2 (6.9)	29 (100.0)
BRCAX	3 (6.1)	30 (61.2)	5 (10.2)	11 (22.4)	49 (100.0)

Table 3 Contingency table of ERβ and clinicopathological parameters in familial breast cancers, cut-off for positive expression at 20% of tumour cells ($n = 123$)

	Nuclear pan-ERβ			Cytoplasmic pan-ERβ			Nuclear ERβ1			Cytoplasmic ERβ1		
	Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value
Grade												
I, II	4	33	0.244	16	21	0.594	6	21	0.909	14	13	0.461
III	13	53		25	41		14	46		26	34	
<2 cm	13	55	0.421	29	39	0.606	14	43	0.691	25	32	0.736
≥2 cm	6	39		17	28		8	30		18	20	
Negative	18	72	0.343	37	53	0..720	18	60	0.846	38	40	0.185
Positive	4	28		12	20		6	18		8	16	
Negative	13	34	0.014*	19	28	0.874	10	33	0.760	18	25	0.432
Positive	6	56		26	36		13	37		25	25	
Negative	14	45	0.060	23	36	0.596	12	41	0.591	24	29	0.832
Positive	5	45		22	28		11	29		19	21	
Negative	18	75	0.234	37	56	0.195	22	61	0.748	38	45	0.536
Positive	0	6		4	2		1	4		3	2	
Not given	14	58	0.743	34	38	0.504	16	47	0.492	26	37	0.281
Given	5	25		12	18		4	18		12	10	
Not given	8	42	0.616	40	10	0.275	8	32	0.537	14	26	0.247
Given	12	49		45	16		13	38		24	27	
RFS			0.087			0.820			0.805			0.260
BCSS			0.382			0.725			0.296			0.179

	Nuclear ERβ2			Cytoplasmic ERβ2		
	Negative	Positive	*P* value	Negative	Positive	*P* value
Grade						
I, II	4	19	0.812	7	16	0.920
III	9	50		17	41	
Size						
<2 cm	8	43	0.702	11	40	0.184
≥2 cm	5	34		13	25	
Nodal status						
Negative	10	63	0.402	20	53	0.778
Positive	5	19		7	16	
ER alpha						
Negative	8	34	0.705	8	34	0..024*
Positive	7	40		19	27	
PgR						
Negative	9	42	0.817	14	37	0.440
Positive	6	32		13	24	
HER2 status						
Negative	13	64	0.317	21	55	0.710
Positive	0	5		1	4	
Endo treat						
Not given	11	49	0.869	22	38	0.207
Given	4	16		4	15	
Chemotherapy						
Not given	7	30	0.422	15	22	0.042*
Given	7	43		10	39	

Table 3 continued

	Nuclear ERβ2			Cytoplasmic ERβ2		
	Negative	Positive	*P* value	Negative	Positive	*P* value
RFS			0.497			0.075
BCSS			0.841			0.041*

Endo treat endocrine treatment, *RFS* relapse-free survival, *BCSS* breast cancer-specific survival

*Significant *P* value < 0.05

grade, lymph node status, plus endocrine treatment and nuclear ERβ1 as interaction variables ($P = 0.203$). No correlation was seen between pan-ERβ, ERβ1 or ERβ2 expression and survival in patients not treated with tamoxifen ($P > 0.05$).

Survival analysis by treatment with chemotherapy

For patients treated with chemotherapy, cytoplasmic ERβ2 expression at a cut-off score of 6, correlated with poorer survival at 15 years ($P = 0.003$) (Table 4; Fig. 1h). At a cut-off 20%, a trend was present, but this did not reach statistical significance ($P = 0.057$) (Fig. 1g). The survival curves at 5 years showed a similar trend although this did not reach statistical significance ($P = 0.159$ at a cut-off score of 6). No such differences were seen in patients not treated with chemotherapy ($P > 0.05$ irrespective of cut-offs used). The significance of cytoplasmic ERβ2 on response to chemotherapy at 15 years was confirmed by multivariate analysis including chemotherapy and non-threshold cytoplasmic ERβ2 (score out of 7) as interaction variables ($P = 0.045$, hazard ratio 1.22, 95% CI 1.004–9.87) (Table 5). This however did not reach statistical significance at a cut-off of 20% ($P = 0.056$, hazard ratio 1.21, 95% CI 1.00–1.48) (Table 5).

Expression of nuclear ERβ1 (cut-off at score of 7) correlated with better 15-year survival in patients treated with chemotherapy ($P = 0.029$), however, this did not reach statistical significance on a multivariate analysis, including grade, lymph node status plus non-threshold ERβ1 score out of 7 and chemotherapy as interaction variables ($P = 0.979$, hazard ratio 1.00, 95% CI 0.83–1.20), or at a cut-off of 20% ($P = 0.339$). There was no correlation between pan-ERβ expression and chemotherapy response ($P > 0.05$).

Discussion

In this series of familial breast cancers nuclear pan-ERβ was expressed in 81% of cases, with no significant difference between the three BRCA1 (77%), BRCA2 (84%) and BRCAX (84%) groups. This is similar to 84% (94% BRCA1, 75% BRCA2 and 81% BRCAX) obtained by Daidone et al. [19] and higher than 42% reported in a series of 48 patients with founder BRCA1 mutation [20]. Although potentially due to differing patient cohorts, the discrepancy with the latter study may be due to their use of a different polyclonal antibody. We have used a monoclonal pan-ERβ antibody that has been validated by others and is likely to reflect true positivity [22]. Indeed, there was no significant variation across the four molecular subtypes in familial breast is in keeping with previous studies using this validated antibody [17].

Recent studies on sporadic cancers using the same antibody clone have yielded discordant results regarding the impact of nuclear ERβ1 on prognosis including: better survival particularly in triple negative cancers and post-menopausal women [16], better survival in node negative luminal A tumours [17], worse survival in node positive

Table 4 Log rank test, 5- and 15-year overall survival, familial breast tumours, stratified by cytoplasmic ERβ2 expression

	5-year overall survival, *P* value		15-year overall survival, *P* value	
	Cut-off 20%	Cut-off score 6 out of 7	Cut-off 20%	Cut-off score 6 out of 7
All familial tumours	0.226	0.061	0.045*	0.002*
Basal-type tumours	0.182	0.039*	0.159	0.011*
BRCA1 tumours	0.372	0.269	0.306	0.068
Tumours treated with chemotherapy	0.195	0.021*	0.057	0.003*
Tumours not treated with chemotherapy	0.754	0.323	0.851	0.734

*Significant *P* value < 0.05

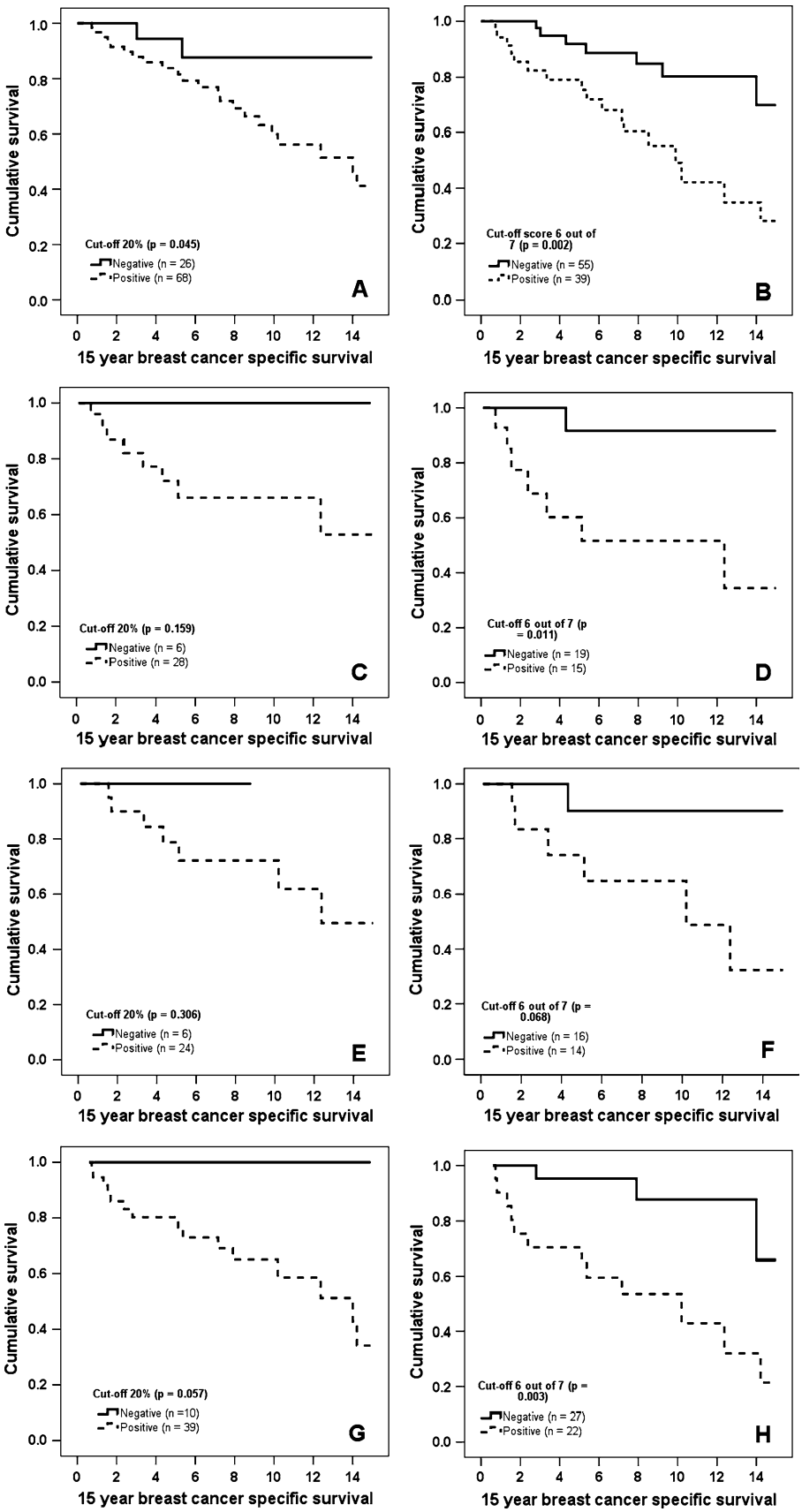

Fig. 1 Kaplan Meier curves of 15 year breast-cancer-specific survival stratified by cytoplasmic ERβ2, cut-off for positivity at a score of 6 out of 7 (**b**, **d**, **f**, **h**), and at 20% (**a**, **c**, **e**, **g**). All familial cancers (**a**, **b**), basal-type cancers (**c**, **d**), BRCA1 cancers (**e**, **f**) and cancers treated with chemotherapy (**g**, **h**)

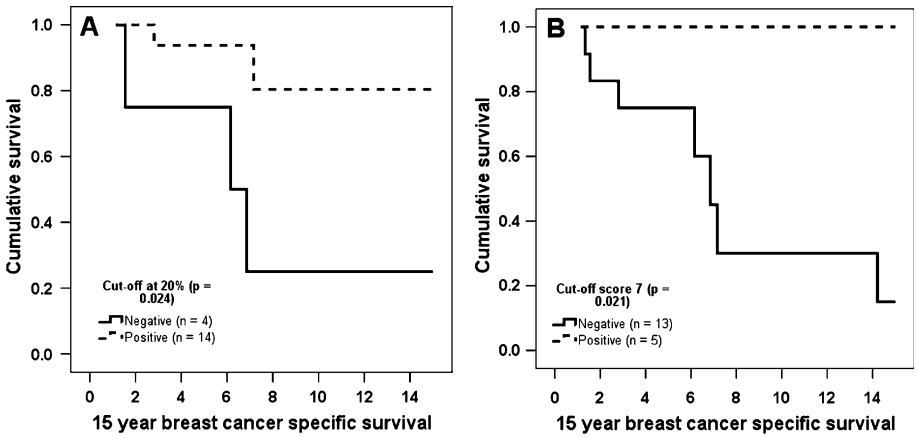

Fig. 2 Kaplan Meier curves of breast-cancer-specific survival at 15 years, treated with endocrine therapy stratified by nuclear ERβ1, cut-offs at 20% (**a**) and score of 6 (**b**)

Table 5 Multivariate analysis using Cox regression model, 15-year overall survival in familial breast cancers, with (a) non-threshold cytoplasmic ERβ2 (score out of 7) and (b) with threshold cytoplasmic ERβ2 (20%) and chemotherapy as interaction variables

	P value	Hazard ratio	95% CI for hazard ratio
(a) Non-threshold cytoplasmic ERβ2 (score out of 7)			
Cytoplasmic ERβ2 * chemotherapy	0.045	1.22	1.004–9.87
Nodal status	0.048	3.83	1.01–14.53
Grade	0.115	3.72	0.73–19.01
(b) Threshold cytoplasmic ERβ2 (20%)			
Cytoplasmic ERβ2 * chemotherapy	0.056	1.21	1.00–1.48
Nodal status	0.012	3.36	1.31–8.62
Grade	0.771	1.22	0.312–4.82

luminal B tumours [17] and no impact on prognosis [18]. In this study of familial cancers, while there was a trend for nuclear ERβ1 to be associated with a better prognosis, this did not reach statistical significance. Again this may be due to different cohorts, cut-offs employed and number of the familial cancers available for our study. Nuclear ERβ1, however, was predictive of response to endocrine therapy at both cut-offs, in concordance with 10 out of 13 previous ERβ studies as reviewed by Fox et al. [27]. This is supported by cell line studies where the induction of ERβ expression enhanced the anti-proliferative effects of tamoxifen [28]. This may have important treatment implications, particularly for BRCA1 cancers, since a significant proportion of these cases was negative for ERα but positive for nuclear ERβ1 (77%, 20/26).

In addition to nuclear expression, in our other studies [25] and in accordance with other investigators [14, 18, 22, 29], we also noted cytoplasmic expression. The cytoplasmic staining present for the ERβ antibody clones used in our study is likely to be specific as it has been terminated by peptide absorption in previous studies [18, 22]. This is in keeping ERα and ERβ having a non-genomic signalling function, and the role of ERα and ERβ in the transcription of mtDNA in the mitochondrion [30, 31]. Indeed, immunoblotting of subcellular fractions have confirmed the presence of ERβ within the nucleus, cytoplasm and the caveolae of plasma membranes [32].

Although there was no significant correlation between either nuclear or cytoplasmic ERβ2 and classical prognostic factors including size, grade and lymph node status it is interesting to note that cytoplasmic positivity was associated with poorer survival at 15 years regardless of cut-offs employed. For basal-type cancers, this was significant at 5 and 15 years, when a cut-off score of 6 was used. A similar trend was seen at a cut-off of 20%, however, this did not reach statistical significance. The absence of a statistical association at this cut-off may be due to the limited number of cases with <20% staining, but increasing the cohort size in familial breast cancer is difficult. Similarly, there was a trend for shorter overall survival in BRCA1 tumours. Overall, these findings are consistent with a previous study by Shaaban et al. [18], where cytoplasmic ERβ2 was associated with a poorer prognosis.

The effect of ERβ on basal-type and BRCA1 cancers noted in our study are supported by cell line studies where the introduction of ERβ into a ERα negative cell line MDA-MB-435 resulted in increased proliferation, invasiveness and metastasis [33]. Whereas in ERα positive cells, the introduction of ERβ led to the inhibition of genes associated with proliferation [27]. Extranuclear ERβ may

have rapid non-genomic effects including stimulation of cell proliferation via G protein, ERK and c-Jun kinase activation [32, 34, 35]. In addition, induction of ERβ-dependent transcription of mtDNA (COXI, COXII and ND1 subunit complex 1) in the mitochondrion may result in alterations in energy metabolism, abnormal growth and inhibition of apoptosis [31, 36–38].

Furthermore, the expression of cytoplasmic ERβ2 in our study was associated with shorter survival at 15 years in patients receiving chemotherapy. This was significant at a cut-off score of 6, and was also significant in a multivariate analysis with non-threshold data (score out of 7) and chemotherapy as interaction variables. Multivariate analysis at a 20% cut-off did not reach statistical significance ($P = 0.056$), however, this may be due to the limited number of tumours with ≤20% expression treated with chemotherapy. Assessment of ERβ2 expression may be of clinical relevance, as a proportion of familial triple negative cancers, which are resistant to targeted therapies, express cytoplasmic ERβ2 in >20% of cells (32/40, 80%).

Chemotherapeutic agents such as cyclophosphamide, doxorubicin and paclitaxel initiate apoptosis by increasing permeability of the mitochondrial membrane, either through induction of p53/bcl-2 expression (secondary to DNA damage), or by the generation of reactive oxygen species [31, 39, 40]. This leads to Ca^{2+} overload of the mitochondrial matrix and dissipation of the electrochemical gradient which drives ATP generation, resulting in swelling and rupture of the mitochondrion, followed by the release of pro-apoptotic proteins [31, 41]. Mitochondrial ERβ may block apoptosis by promoting transcription of respiratory chain protein mt-DNA (such as subunits of ATP synthase, complex III and IV), leading to increased ATP production and the neutralisation of reactive oxygen species [31]. Inhibition of apoptosis may also occur via direct inhibition of the Ca^{2+} uniporter by mitochondrial estrogen receptors [42]. The role of ERβ in the inhibition of apoptosis is further supported by the lowering of resting mitochondrial membrane potential following mitochondrial ERβ knockdown [43]. Chemotherapy resistance may be further enhanced by the rapid non-genomic effects of extranuclear ERβ on cell proliferation [31].

The correlation between cytoplasmic ERβ2, but not ERβ1 with survival provides further evidence to support the different transactivating properties of the different ERβ isoforms [44]. While ERβ1 is the only fully functional isoform and may form ERβ1 homodimers, ERβ2 to 5 cannot form homodimers, but may form heterodimers with ERβ1 only. Under the stimulation of estrogens, ERβ1 preferentially forms heterodimers with ERβ2-5 with enhanced transactivating properties when compared to ERβ1 homodimers [44].

A correlation between loss of ERα protein and BRCA1 mutation has been reported [5, 45, 46]. There are limited data on the relationship between ERβ and BRCA1, however, it is known under the influence of the phytoestrogen genistein, BRCA1 inhibits ERβ but not ERα reporter activity [47]. The presence of BRCA1 mutations may therefore enhance ERβ activity in ERα negative tumours.

In summary, this is the first study to comprehensively analyse the subcellular expression of ERβ and its different isoforms in familial breast cancer. Our study highlights the impact cytoplasmic ERβ2 on prognosis and response to treatment. Since it has been reported that 5–10% of patients with ERα negative breast cancer respond to tamoxifen [7, 48] and that this effect is may be predicted by ERβ expression, the clinical diagnostic measurement of nuclear ERβ1 may identify patients with ERα negative tumours that may benefit from endocrine therapy.

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