Prognostic Value of ER and PgR Expression and the Impact of Multi-clonal Expression for Recurrence in Ductal Carcinoma *in situ*: Results from the UK/ANZ DCIS Trial

Mangesh A. Thorat[1,2,3], Pauline M. Levey[4], J. Louise Jones[5], Sarah E. Pinder[2,6], Nigel J. Bundred[7,8], Ian S. Fentiman, and Jack Cuzick[1]

ABSTRACT

Purpose: The prognostic value of estrogen receptor (ER)/progesterone receptor (PgR) expression in ductal carcinoma *in situ* (DCIS) is unclear. We observed multi-clonality when evaluating ER/PgR expression in the UK/ANZ DCIS trial, therefore, we investigated the prognostic role of both uni-clonal and multi-clonal ER/PgR expression in DCIS.

Experimental Design: Formalin-fixed paraffin embedded tissues were collected from UK/ANZ DCIS trial participants ($n = 755$), and ER/PgR expression was evaluated by IHC in 181 cases (with recurrence) matched to 362 controls by treatment arm and age. Assays were scored by the Allred method and by a newly devised clonal method—analyses categorizing multi-clonal DCIS as ER/PgR-positive as per current practice (Standard) and as ER/PgR-negative (clonal) were performed.

Results: ER expression was multi-clonal in 11% (39/356) of ER-positive (70.6%, 356/504) patients. Ipsilateral breast event (IBE) risk was similarly higher in ER-multi-clonal and ER-negative DCIS as compared with DCIS with uni-clonal ER expression. ER-negative DCIS (clonal) had a higher risk of *in situ* IBE [OR 4.99; 95% confidence interval (CI), 2.66–9.36; $P < 0.0001$], but the risk of invasive IBE was not significantly higher (OR 1.72; 95% CI, 0.84–3.53; $P = 0.14$), $P_{heterogeneity} = 0.03$. ER was an independent predictor in multivariate analyses (OR 2.66; 95% CI, 1.53–4.61). PgR status did not add to the prognostic information provided by ER.

Conclusions: ER expression is a strong predictor of ipsilateral recurrence risk in DCIS. ER-positive DCIS with distinct ER-negative clones has a recurrence risk similar to ER-negative DCIS. ER should be routinely assessed in DCIS, and ER scoring should take clonality of expression into account.

Introduction

Estrogen receptor (ER) expression is a well-established prognostic and predictive biomarker for invasive breast cancer. The prognostic and predictive role of ER expression in ductal carcinoma *in situ* (DCIS) is less clear and consequently it is not routinely evaluated in DCIS in many parts of the world (1, 2).

Prognostic and predictive factors both play important role in treatment of cancers, with the former predicting the risk of recurrence and thereby guiding intensity of therapeutic approach including surgical treatment and the latter assisting in selection of adjuvant treatment(s). In patients with DCIS where breast conservation is feasible, adjuvant treatment is currently mainly guided by grade and tumor size. However, wide variations in practice remain among different regions of the world, including variations in ER evaluation (1, 2) and use of adjuvant endocrine therapy. For example, European Society of Medical Oncology guidelines (3) recommend adjuvant radiotherapy in low/intermediate grade DCIS larger than 10 mm, whereas many centers do not routinely offer radiotherapy in any low/intermediate grade DCIS or in tumors smaller than 25 mm, similar to patients in the cohort 1 of E5194 trial (4) in the United States. The 12-year ipsilateral breast event (IBE) rate without radiotherapy was 14.4% in this E5194 cohort (4), which is neither high nor low. A prognostic factor independent of grade and tumor size can further stratify such patients in low- and high-risk groups to aid adjuvant radiotherapy decisions. Furthermore, a prognostic factor can also aid in surgical decisions. For example, a patient with a high-grade DCIS lesion requiring level II oncoplastic procedure with complex localization and a high chance of margin involvement may opt for a mastectomy with immediate breast reconstruction and avoid radiotherapy altogether if an independent prognostic factor predicts even higher IBE risk. Common molecular prognostic and predictive factors in invasive breast cancer, namely, ER, progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) may fulfill such a role and merit further research to evaluate their role in DCIS.

[1]Centre for Cancer Prevention, Wolfson Institute of Preventive Medicine, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom. [2]School of Cancer & Pharmaceutical Sciences, Faculty of Life Sciences & Medicine, King's College London, London, United Kingdom. [3]Breast Services, Guy's Hospital, Guy's and St Thomas' NHS Foundation Trust, Great Maze Pond, London, United Kingdom. [4]Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom. [5]Barts Cancer Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom. [6]Department of Pathology, Guy's Hospital, Guy's and St Thomas' NHS Foundation Trust, Great Maze Pond, London, United Kingdom. [7]Manchester University NHS Foundation Trust, Wythenshawe, Manchester, United Kingdom. [8]Division of Cancer Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, United Kingdom.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Prior presentations: Some findings presented as a poster at the San Antonio Breast Cancer Symposium 2015.

Corresponding Author: Mangesh A. Thorat, Centre for Cancer Prevention, Wolfson Institute of Preventive Medicine, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, United Kingdom. Phone: 44 0207 882 3546; Fax: 44 0207 882 3890; E-mail: m.thorat@qmul.ac.uk, thoratmangesh@gmail.com

Clin Cancer Res 2021;27:2861–7

doi: 10.1158/1078-0432.CCR-20-4635

Translational Relevance

The prognostic role of estrogen receptor (ER)/progesterone receptor (PgR) in ductal carcinoma *in situ* (DCIS) remains unclear, and ER/PgR are not routinely evaluated in DCIS in many parts of the world. Our study design eliminated treatment allocation bias and treatment-related confounding to robustly assess the true prognostic value of ER/PgR in DCIS. We observed multi-clonality in ER expression in 11% of ER-positive DCIS, that is, ER-positive DCIS with distinct ER-negative clones, and investigated the prognostic role of such multi-clonality in ER expression.

We report that ER is a strong prognostic factor with greater than 3-fold risk of ipsilateral recurrence in ER-negative DCIS. Therefore, ER should be routinely assessed in DCIS and the clonal method reported here should be considered. Furthermore, multi-clonality in DCIS suggests multi-clonal DCIS as an excellent model to investigate cancer progression and tumor evolution as well as therapeutic effectiveness and resistance, particularly in relation to the use of targeted therapies.

Twenty-six studies (Supplementary references S1–S26) have investigated the prognostic role of ER in DCIS; however, only five of these (5–9) found a statistically significant lower risk of IBE in ER-positive cases. Allred and colleagues (10) assessed the role of ER in predicting benefit of adjuvant tamoxifen in the NSABP-B24 trial. They determined ER status in 732 (40.6%) participants by central ($n = 449$; 24.9%) or local ($n = 283$; 15.7%) assessment. Tamoxifen reduced recurrences by 42% in ER-positive DCIS [HR = 0.58; 95% confidence interval (CI), 0.42–0.81] but not in ER-negative DCIS (HR = 0.88; 95%CI, 0.49–1.59). These results suggesting predictive role of ER were based on combining central and local institutional assessments with only 74.5% agreement (positive vs. negative) in a subset of patients ($n = 102$) with both assessments.

Only two of 17 studies (Supplementary references S1–S7, S10, S12–S14, S16–S20, S22, S23, S26, S27) investigating the relationship between PgR expression and recurrence in DCIS reported a statistically significant relationship (6, 8). In the NSABP-B24 study (10), PgR expression was predictive of tamoxifen benefit, but was not more informative than ER.

The lack of significant association between ER/PgR expression and IBE risk in most studies is unsurprising given their limitations, such as small sample size and treatment-related confounding. ER expression is inversely associated with adverse histologic features in DCIS (11) and this may result in treatment bias, for example, more frequent use of adjuvant radiotherapy or mastectomy in ER-negative DCIS, thus confounding the true association between ER and IBE risk. Therefore, the prognostic role of ER/PgR expression in DCIS is still unclear and a well-designed study that overcomes these limitations is needed. This is best achieved through a randomized trial with long-term follow-up. We evaluated prognostic value of ER/PgR expression in DCIS in a case–control study within in the UK/ANZ DCIS trial (12). During the course of this study, we observed multi-clonal ER/PgR expression in DCIS and assessed the prognostic relevance of multi-clonality. This led to a new method for assigning ER/PgR status—designated as the clonal method.

Materials and Methods

Study population

The study samples were derived from the UK/ANZ DCIS trial participants (12); a randomized 2×2 factorial design trial investigating roles of tamoxifen and/or radiotherapy as adjuvant treatment in DCIS. The trial enrolled a total of 1,694 patients (Supplementary Fig. S1) and median follow-up is 12.7 years (12). Pathology material collection and use in biomarker studies was approved by the National Research Ethics Service—Joint UCL/UCLH Committees on Ethics of Human Research.

Formalin-fixed paraffin embedded (FFPE) tissue blocks containing DCIS were collected from 36 hospitals in the United Kingdom. The subset, labelled as biomarker study subset 1 (BSS1) hereafter, comprised 755 (45%) patients, it was similar to the remaining trial population with regard to treatment allocation and clinicopathologic factors including age and completeness of excision but contained a significantly higher proportion of high-grade tumors, and large tumors with necrosis (Supplementary Table S1).

Study design

A case–control study (below) was nested within the BSS1 to eliminate any residual treatment-related confounding. Study sample size was not formally estimated, and ER/PgR expression was evaluated in all case-control samples. This study is reported in accordance with the REMARK criteria (13).

Case–control matching

Controls (participants without recurrence) were individually matched (1:2 case–control ratio) to cases (participants with *in situ* or invasive recurrence) by age ± 7 years and treatment allocation; controls had to be followed up for at least the same length of time as the time to event in their matching case (Supplementary Materials and Methods). The nested case–control study comprised 181 cases and 362 controls (**Table 1**).

IHC assays and evaluation of ER and PgR

ER and PgR IHC assays were performed on whole sections using mouse mAbs 1D5 (Dako) and PGR636 (Dako), respectively (14, 15), and the EnVision FLEX+ detection system (Dako UK Ltd.) on a Dako Autostainer (Dako).

Assays were scored by the Allred method (16), and H-scores (17) were also recorded. Multi-clonal nature of ER/PgR expression (Supplementary Fig. S2) was observed and a new scoring method—designated the "clonal method," was devised. ER or PgR-positive DCIS was labeled as ER-multi-clonal or PgR-multi-clonal if it showed presence of at least one carcinoma *in situ* duct with a complete lack of ER or PgR expression (independent of different DCIS components such as morphologic patterns and grades). Under the hypothesis that clonal subpopulations of cancer cells completely lacking ER or PgR expression are the dominant clones with a greater impact on outcome, such multi-clonal DCIS was assigned ER/PgR-negative status by the clonal method. In short, current methods (e.g., Allred, Quick score) classify ER-multi-clonal DCIS as ER positive, whereas the clonal method classifies it as ER negative.

Clinicopathological variables

Data on clinicopathological variables were derived from the trial database and a pathology review (18). Age, completeness of excision, treatment allocation, tumor size (in mm), cytonuclear grade (UK National Pathology Group; ref. 19), necrosis and periductal inflammation were analyzed.

Statistical analysis

Trial procedures, follow-up (12), and a histopathology review (18) have been reported previously. For these analyses, only the first

Table 1. Clinicopathological characteristics of patients included in the case–control study.

Variable Patients	Strata *N*	Cases 181	Controls 362	χ^2	*P*
Age	Median (IQR) [years]	56.9 (52.9–61.2)	57.0 (53.5–61.5)	0.10	0.75[a]
Tumor size	Median (IQR) [mm]	16.0 (12–22) ($n = 166$)	14.0 (8.5–19) ($n = 319$)	13.07	**0.0003**[a]
Grade	Low	5 (2.8%)	22 (6.1%)	7.50	**0.023**
	Intermediate	17 (9.4%)	53 (14.6%)		
	High	142 (78.5%)	240 (66.3%)		
	No information	17 (9.4%)	47 (13.0%)		
Necrosis	Absent	6 (3.3%)	31 (8.6%)	5.94	**0.015**
	Present	160 (88.4%)	284 (78.5%)		
	No information	15 (8.3%)	47 (13.0%)		
Inflammation	Absent	21 (11.6%)	68 (18.8%)	6.15	**0.013**
	Present	144 (79.6%)	240 (66.3%)		
	No information	16 (8.8%)	54 (14.9%)		
Excision	Incomplete	37 (20.4%)	43 (11.9%)	7.91	**0.019**
	Uncertain	29 (16.0%)	49 (13.5%)		
	Complete	102 (56.4%)	236 (65.2%)		
	No information	13 (7.2%)	34 (9.4%)		
ER	Negative	73 (40.3%)	75 (20.7%)	31.83	<**0.0001**
	Multiclonal	19 (10.5%)	20 (5.5%)		
	Positive	78 (43.1%)	239 (66.0%)		
	No information	11 (6.1%)	28 (7.7%)		
PgR	Negative	77 (42.5%)	86 (23.8%)	26.12	<**0.0001**
	Multiclonal	33 (18.2%)	51 (14.1%)		
	Positive	59 (32.6%)	192 (53.0%)		
	No information	12 (6.6%)	33 (9.1%)		
RT	Not given	140 (77.4%)	280 (77.4%)	0	1
	Given	41 (22.7%)	82 (22.7%)		
Tamoxifen	Not given	97 (53.6%)	194 (53.6%)	0	1
	Given	84 (46.4%)	168 (46.4%)		

Abbreviation: IQR, interquartile range.
[a]Wilcoxon rank-sum test; missing values, although given in "No Information" row, are not treated as a separate category. Values in bold are statistically significant.

recurrence event was considered. Missing data were not imputed. Associations between a continuous and an ordinal variable were assessed by the Kruskal–Wallis test and those between two ordinal variables by Goodman–Kruskal gamma. Analyses of the risk of recurrence by groups were performed using conditional logistic regression model to estimate matched ORs (mOR) with 95% CI. These univariate [ER or PgR or hormone receptor (HR)] and multivariate analyses (additional covariates tumor size, grade, completeness of excision, presence of necrosis, and presence of inflammation) used any IBE as the primary case definition. Analyses with *in situ* IBE (DCIS-IBE) and invasive IBE (I-IBE) as case definitions were also performed.

Analyses using the Allred method are labeled as ER (standard)/PgR (standard) and those using the clonal method are labelled as ER (clonal)/PgR (clonal). Sensitivity analyses separating ER or PgR status into three categories, namely, pure positive, multi-clonal, and negative were also performed.

All *P* values are two sided and values ≤0·05 were deemed significant. Comparisons of different models were based on differences in χ^2 values of respective likelihood ratio tests. Statistical analyses were performed using STATA 13.0 (StataCorp LP). Kaplan–Meier survival plots weighted for control sampling in the BSS1 were generated using NestedCohort (20, 21) package (version 1.1–3) in R (version 3.3.2; R Foundation for Statistical Computing).

Table 2. Univariate analysis of ER status (standard and clonal) as a predictor of recurrence.

		Standard method			Clonal method		
		mOR (95%CI)			mOR (95%CI)		
Case definition	Events[a]	ER-negative *n* = 148	ER-positive *n* = 346	*P*	ER-negative *n* = 187	ER-positive *n* = 307	*P*
IBE	138	2.81 (1.76–4.48)	0.36 (0.22–0.57)	<0.0001	3.33 (2.09–5.30)	0.30 (0.19–0.48)	<0.0001
I-IBE	51	1.53 (0.71–3.26)	0.66 (0.31–1.40)	0.28	1.72 (0.84–3.53)	0.58 (0.28–1.20)	0.14
DCIS-IBE	85	3.85 (2.09–7.08)	0.26 (0.14–0.48)	<0.0001	4.99 (2.66–9.36)	0.20 (0.11–0.38)	<0.0001

Note: The mORs presented in this table are in both directions, that is, with ER-positive as reference in the first column and ER-negative as reference in the second column to permit easier comparisons with the published literature.
Abbreviation: mOR, matched odds ratio.
[a]Recurrence type not known in two cases.

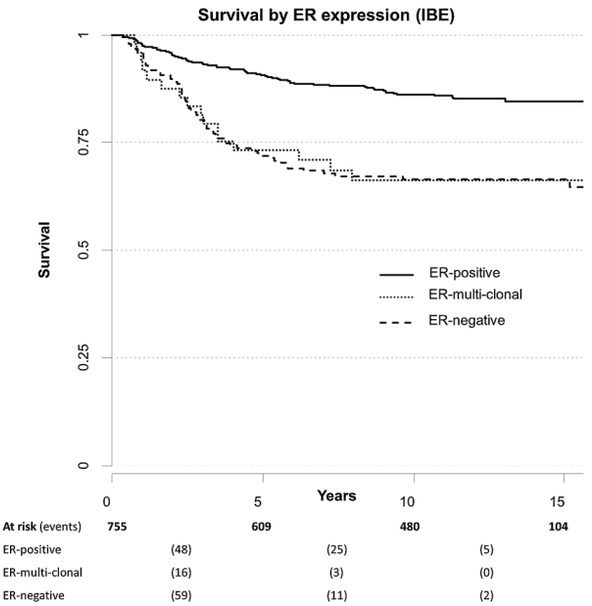

Figure 1.
Kaplan–Meier survival plots for IBE by ER expression in DCIS with ER multi-clonal cases plotted as a separate category. Plots are weighted for control sampling in the BSS1 using NestedCohort (20, 21) package.

Result

ER expression in DCIS

One-hundred and forty-eight (29.4%) of 504 evaluable tumors were ER negative and 356 (70.6%) were ER positive (**Table 1**) with 39 (11%) of these displaying multi-clonal ER expression (Supplementary Fig. S2; Supplementary Table S2), median Allred score in ER-multi-clonal cases was 7 [interquartile range (IQR), 5–8] and 15 (38.5%) cases had Allred score of 8. Thirty-nine samples were not evaluable (see Supplementary Materials and Methods).

ER positivity was associated with decreasing age ($P = 0.011$), tumor size ($P = 0.0009$), cytonuclear grade (19; ref. $P < 0.0001$), necrosis ($P < 0.0001$), and periductal inflammation ($P < 0.0001$; Supplementary Table S3). ER correlated strongly with PgR ($\chi^2 = 62.49$; $P < 0.0001$); 31 (79.5%) ER-multi-clonal cases had multi-clonal PgR expression, 5 (12.8%) were PgR negative and 3 (7.7%) were PgR positive.

ER status and the risk of recurrence

ER-negative (standard) DCIS had an almost 3-fold higher risk of ipsilateral recurrence (mOR = 2.81; 95% CI, 1.76–4.48) in univariate analyses, mainly driven by a higher DCIS-IBE risk (**Table 2**). The risk of I-IBE was nonsignificantly increased. However, the increase in I-IBE risk was not significantly different than that in DCIS-IBE risk ($P_{\text{heterogeneity}} = 0.06$). Prognostic discrimination was higher when ER status was determined by the clonal method [$\Delta\chi^2$ 8.64 (IBE), 9.47 (DCIS-IBE), 1.03 (I-IBE)] and the increase in DCIS-IBE risk (mOR = 4.99; 95% CI, 2.66–9.36) was significantly higher ($P_{\text{heterogeneity}} = 0.03$) than the increase in I-IBE risk (mOR = 1.72; 95% CI, 0.84–3.53). ER-negative (clonal) DCIS had a 5-fold higher risk of DCIS-IBE. With ER-negative DCIS as reference, the OR for multi-clonal DCIS was very close to unity (**Table 3**). Thus, while ER-multi-clonal DCIS cases are classified as ER positive by current methods, these lesions behave much more like ER-negative DCIS in terms of high recurrence risk. Survival curves for ER-negative and ER-multi-clonal DCIS were very similar in Kaplan–Meier survival plots (**Fig. 1**). Allred score (continuous variable) was a predictor of IBE risk (mOR = 0.88; 95% CI, 0.83–0.93); however, addition of ER (clonal) significantly improved the model [$\Delta\chi^2$ (1d.f.) 9.33; $P = 0.0023$]. ER-positive (clonal) status (mOR = 0.27; 95% CI, 0.11–0.63) and not the Allred score (mOR = 1.02; 95% CI, 0.91–1.15) was an independent predictor in this model confirming that the association between IBE risk and multi-clonal ER expression is independent of Allred score.

The role of ER expression in multivariate analyses

Inclusion of ER (standard) in a multivariate model including clinicopathological variables (tumor size, grade, completeness of excision, presence of necrosis, and presence of inflammation—null model) significantly improved the model [$\Delta\chi^2$ (1d.f.) 10.55; $P = 0.0012$] and ER-negative (standard) status was an independent predictor of recurrence (mOR = 2.42; 95% CI, 1.40–4.19; $P = 0.0016$). The improvement in model was greater [$\Delta\chi^2$ (1d.f.) 13.28; $P = 0.0003$] with ER (clonal) (**Table 4**). Analyses with multi-clonal DCIS as a separate category show similar results (Supplementary Table S4).

Table 3. ER status as a predictor of recurrence when multi-clonal DCIS is categorized separately.

Case definition	Events[a]	Subgroup	mOR (95% CI)	*P*	mOR (95% CI)	*P*
IBE	138	ER-positive[b]	1 (reference)	—	0.30 (0.18–0.49)	<0.0001
		ER-multi-clonal	3.23 (1.48–7.04)	0.0033	0.96 (0.44–2.13)	0.93
		ER-negative	3.36 (2.04–5.51)	<0.0001	1 (reference)	—
I-IBE	51	ER-positive[b]	1 (reference)	—	0.61 (0.28–1.34)	0.22
		ER-multi-clonal	2.14 (0.54–8.49)	0.28	1.31 (0.30–5.69)	0.72
		ER-negative	1.63 (0.75–3.55)	0.22	1 (reference)	—
DCIS-IBE	85	ER-positive[b]	1 (reference)	—	0.20 (0.10–0.38)	<0.0001
		ER-multi-clonal	4.66 (1.73–12.54)	0.002	0.92 (0.34–2.44)	0.86
		ER-negative	5.09 (2.61–9.89)	<0.0001	1 (reference)	—

Note: The mORs presented in this table are in both directions, that is, with ER-positive (uni-clonal) as reference in the first column and ER negative as reference in the second column.
Abbreviation: mOR, matched odds ratio.
[a]Recurrence type not known in two cases.
[b]Uni-clonal ER-positive—DCIS not exhibiting multi-clonal expression.

Table 4. Multivariate analysis of ER (clonal), other tumor characteristics, and completeness of excision as a prognostic factor for IBE.

			Univariate[a]		Multivariate	
Variable	**Subgroup**	***N***	**mOR (95% CI)**	***P***	**mOR (95% CI)**	***P***
ER (clonal)	Positive	188	1 (reference)	—	1 (reference)	—
	Negative	125	2.93 (1.76–4.87)	<0.0001	2.66 (1.53–4.61)	0.0005
Excision	Complete	198	1 (reference)		1 (reference)	
	Uncertain	60	1.30 (0.69–2.44)	0.42	1.28 (0.65–2.50)	0.48
	Incomplete	55	1.48 (0.80–2.72)	0.21	1.41 (0.71–2.77)	0.32
	Trend test			*0.17*		*0.25*
Size (mm)		313	1.03 (1.00–1.06)	0.026	1.02 (1.00–1.05)	0.10
Grade	Low	17	1 (reference)		1 (reference)	
	Intermediate	42	1.35 (0.38–4.80)	0.64	0.96 (0.23–3.93)	0.95
	High	254	1.88 (0.61–5.83)	0.27	0.90 (0.23–3.54)	0.88
	Trend test			*0.17*		*0.84*
Necrosis	No	22	1 (reference)		1 (reference)	
	Yes	291	1.54 (0.58–4.10)	0.39	1.12 (0.37–3.42)	0.84
Inflammation	No	54	1 (reference)		1 (reference)	
	Yes	259	1.84 (0.94–3.60)	0.075	1.19 (0.51–2.80)	0.69

Abbreviations: mOR, matched odds ratio; $n = 313$; events = 117.

[a]Univariate analyses restricted to the same sample size as available for multivariate analyses. Case definition—IBE. Age was a matching variable in the case–control study, and therefore its prognostic impact cannot be assessed.

PgR expression in DCIS

One-hundred and sixty-three (32.7%) of 498 evaluable tumors were PgR negative and 335 (67.3%) were PgR positive with 84 (25%) of these displaying multi-clonal PgR expression (median Allred score 6; IQR 5–7).

The risk of *in situ* (mOR = 3.34; 95% CI, 1.86–6.00; $P < 0.0001$) and any ipsilateral recurrence (mOR = 2.45; 95% CI, 1.55–3.88; $P = 0.0001$) was higher in PgR-negative than PgR-positive DCIS (Supplementary Table S5) but the increase in the risk was not as high as for ER-negative DCIS. Unlike ER, prognostic discrimination was also only marginally higher when PgR status was determined by clonal method ($\Delta\chi^2$ 1.19 for IBE). PgR was not an independent predictor in multivariate models of clinicopathological variables with ER. Inclusion of PgR did not significantly improve these models (Supplementary Table S6).

HR status

HR status (HR negative only if both receptors negative) was a predictor of risk of recurrence (Supplementary Table S7), the risk of *in situ* (mOR = 4.07; 95% CI, 2.17–7.63; $P < 0.0001$) and any ipsilateral recurrence (mOR = 2.88; 95% CI, 1.79–4.63; $P < 0.0001$) was higher in HR-negative DCIS but the models of HR status were not more informative than models using ER status alone (Supplementary Table S8).

Discussion

We assessed the prognostic value of ER/PgR expression in DCIS using a case–control study within the UK/ANZ DCIS trial (12). By the virtue of random treatment allocation and control matching by treatment allocation, we were able to eliminate any treatment allocation bias or treatment-related confounding and robustly assess the true prognostic value of ER/PgR expression in DCIS.

ER-negative DCIS carried a significantly higher risk of *in situ* and overall ipsilateral recurrences. The risk of invasive ipsilateral recurrence was also nonsignificantly higher in ER-negative DCIS. Our findings of a 64% lower IBE risk in ER-positive DCIS (mOR = 0.36) are similar to meta-analysis by Wang and colleagues (22) who reported a significantly lower IBE risk in ER-positive DCIS [relative risk (RR) = 0.39]. Zhang and colleagues (23) reported a nonsignificant 26% lower I-IBE risk in ER-positive DCIS (RR = 0.74) in their meta-analysis, which was similar to our nonsignificantly lower I-IBE risk (mOR = 0.66).

Of note was the importance of multi-clonal expression in 39 (11%) ER-positive DCIS; 30 (77%) of these with an Allred score of ≥5, and 15 (38.5%) with score 8. The prognostic value of multi-clonal ER expression was independent of Allred score. The importance of branched evolutionary tumor growth and resulting intratumor heterogeneity is now increasingly recognized in a variety of cancers (24, 25). However, to the best of our knowledge, multi-clonality in ER/PgR expression in DCIS has not been reported before. This novel observation not only has bearing on prognosis and treatment of DCIS, but also highlights the potential of *in situ* disease models for research into cancer development, progression, and therapeutic resistance. Clonal populations with distinct temporal and/or spatial evolution coalesce and become admixed together due to stromal invasion in invasive cancers, but these remain geographically compartmentalized within individual ducts in DCIS. Therefore, DCIS offers an opportunity to easily identify, separate and investigate different clonal populations, including their impact on outcomes and progression.

Clonal populations with differential molecular target expression are likely to respond differently to targeted therapies. Such differential therapeutic selection pressure resulting in elimination of treatment-responsive clones may make treatment-resistant clones (e.g., without target expression) dominant, and thus adversely impact outcome despite using an effective therapy (26). Our novel observation therefore suggests that an *in situ* disease model would be valuable in investigating therapeutic effectiveness/resistance. The number of multi-clonal cases in our study are too small to draw any treatment-effect inferences (Supplementary Table S9). However, a randomized trial of adjuvant endocrine therapy versus not in ER-multi-clonal DCIS would help investigate such a question but may not be feasible.

Outcomes in ER-multi-clonal DCIS would depend on whether the ER-expressing or the ER-lacking population is the dominant outcome-determining clonal population; and accordingly, the current standard method or the new clonal method would respectively be more

appropriate for scoring of ER. Our observation that outcomes in ER-multi-clonal DCIS are similar to ER-negative DCIS indicates that the ER-lacking clonal population is the dominant outcome-determining population and therefore the new clonal method would be more appropriate for scoring of ER because it more accurately estimates the true prognostic value of ER by avoiding misclassification.

Clinicopathological variables such as age, tumor size, grade, necrosis, and completeness of excision (margin status) are currently the most commonly used prognostic factors. Multivariate analyses showed that inclusion of ER resulted in a significant improvement of the multivariate model of clinicopathologic variables, with a greater improvement using ER (clonal) than ER (standard) and ER was an independent prognostic factor in this model.

The Kaplan–Meier plots (**Fig. 1**) adjusted for control sampling in the entire FFPE subset (36% received radiotherapy) show 10-year ipsilateral recurrence rate of approximately 35% in ER-negative DCIS. Despite all participants receiving radiotherapy, the 10-year IBE rate (21%) in ER-negative DCIS in the NSABP-B24 trial (10) also exceeds the often-recommended threshold of 20% (27) to warrant discussion regarding mastectomy as a surgical option and therefore routine evaluation of ER is merited for its prognostic value alone.

PgR status was a predictor of IBE and DCIS-IBE but (**Table 2**) not an independent predictor in multivariate analyses (Supplementary Table S5). In their meta-analyses, Wang and colleagues (22) reported a nonsignificant 44% lower risk of IBE (RR = 0.56) in PgR-positive DCIS whereas we observed a significantly lower risk of IBE (mOR 0.41). Zhang and colleagues (23) reported a lower risk of I-IBE in PgR-positive DCIS (RR = 0.89) in their meta-analysis, but similar to our results (mOR = 0.84), their results were not statistically significant. Our results show that although PgR is a prognostic factor in DCIS, it does not add further to the information already provided by ER. HR status was also not more informative than ER. Assessment of PgR status by clonal method did not substantially improve the prognostic value of PgR. Among PgR-multi-clonal cases, recurrence rate was lower in ER-positive (16/46, 34.8%) than ER-multi-clonal (15/31, 48.4%) cases. This may suggest that the outcome is mainly driven by ER expression upstream, a likely explanation for the lack of further contribution by PgR to the prognostic information already provided by ER.

Strengths and limitations

A large sample size, random treatment allocation, and good long-term follow-up are major strengths of our study. A careful case–control design further allowed us to eliminate any residual treatment-related confounding. Selection bias however remains one of the limitations of our study. The BSS1 and the case–control study respectively comprised of 45% and 32% of patients enrolled in the trial. Several characteristics of participants in this subset were similar to those in remaining trial participants; however, tumors in the subset were marginally larger and a higher proportion of these were high grade. Furthermore, the proportion of high-grade tumors (68%) in our study is higher compared with other large datasets (18, 28) although not substantially higher than contemporary UK reports (57%; ref. 29). Recruitment in the UK/ANZ DCIS trial started in 1989, almost immediately after initiation of the breast screening programme in the United Kingdom. This resulted in more enrolment of advanced lesions being found in the prevalence screening round. In addition, even though the trial entry criteria (Supplementary Materials and Methods) clearly required patients with only completely excised DCIS to be enrolled, a proportion of cases (Supplementary Table S1) were found to have incomplete or uncertain excision on central pathology review. However, this deviation did not affect our estimation of prognostic value of ER because sensitivity analyses excluding cases with incomplete or uncertain excision showed only minor and nonsignificant changes in effect sizes (Supplementary Materials and Methods). The novel observation of multi-clonal expression is based on 16 events in 39 cases and merits cautious interpretation. The clonal method will be easily transferrable but may slightly increase reporting time. The principal objective of our study was to evaluate prognostic role of ER/PgR, therefore matching of cases and controls by treatment allocation was necessary. As a result, the role of these receptors in predicting tamoxifen treatment benefit could not be assessed in this case–control study; this will be analyzed in further ongoing study.

Conclusions

ER status is not routinely assessed in DCIS (1, 2). Our results show that ER is a strong prognostic factor in DCIS with the risk of ipsilateral recurrence being more than 3-fold higher in ER-negative DCIS. Allred and colleagues (10) suggested the role of ER in predicting tamoxifen benefit. Our results and those from the NSABP-B24 trial (10), together make a strong case for routine evaluation of ER expression in DCIS. Furthermore, a high 10-year IBE risk in ER-negative DCIS in both these studies also warrants that the treatment discussions with patients should also include mastectomy as a surgical option. ER expression was multi-clonal in 11% of ER-positive DCIS in this study and while these cases have recurrence risk similar to that in ER-negative DCIS, they currently get misclassified as ER positive. Therefore, the clonal method reported here should be considered when determining ER status in clinical settings and in future research studies. Our novel observation of multi-clonality in DCIS suggests DCIS as an excellent model to investigate cancer progression, therapeutic effectiveness and resistance, particularly in relation to the use of targeted therapies.

Authors' Disclosures

M.A. Thorat reports grants from Cancer Research UK and Breast Cancer Research Foundation during the conduct of the study and nonfinancial support from Roche outside the submitted work. S.E. Pinder reports grants from Cancer Research UK and the Australian National Health and Medical Research Council during the conduct of the study. J. Cuzick reports grants from Cancer Research UK and Breast Cancer Research Foundation during the conduct of the study. No disclosures were reported by the other authors.

Disclaimer

Funders had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the article for publication. M.A. Thorat had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Authors' Contributions

M.A. Thorat: Conceptualization, formal analysis, funding acquisition, investigation, methodology, writing–original draft, project administration, writing–review and editing. **P.M. Levey:** Data curation, investigation, methodology. **J.L. Jones:** Data curation, supervision, investigation, visualization, methodology. **S.E. Pinder:** Data curation, investigation, writing–review and editing. **N.J. Bundred:** Data curation, writing–review and editing. **I.S. Fentiman:** Data curation, writing–review and editing. **J. Cuzick:** Data curation, formal analysis, supervision, funding acquisition, methodology, writing–review and editing.

Acknowledgments

This work was supported by Amar Ahmad for help with analyses using Nested-Cohort package (version 1.1-3) in R.

This work was funded by Cancer Research UK grants: C569/A12061 (to J. Cuzick, M.A. Thorat) and C569/A16891 (to J. Cuzick, M.A. Thorat).

The Breast Cancer Research Foundation, New York, NY, provided support for biospecimen collection and management activities (J. Cuzick, M.A. Thorat).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 29, 2020; revised January 25, 2021; accepted March 5, 2021; published first March 16, 2021.

References

1. Thomas J, Hanby A, Pinder S, Ellis I, Macartney J, Clements K, et al. Implications of inconsistent measurement of er status in non-invasive breast cancer: a study of 1,684 cases from the sloane project. Breast J 2008;14:33–8.
2. Jarosek S, Tuttle TM, Durham S, Virnig BA. Prognostic factor testing among older women with ductal carcinoma *in situ* and early invasive breast cancer: Data Points # 15. Data Points Publication Series. Rockville (MD): Agency for Healthcare Research and Quality (US); 2011.
3. Cardoso F, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rubio IT, et al. Early breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-updagger. Ann Oncol 2019;30:1194–220.
4. Solin LJ, Gray R, Hughes LL, Wood WC, Lowen MA, Badve SS, et al. Surgical excision without radiation for ductal carcinoma *in situ* of the breast: 12-year results from the ECOG-ACRIN E5194 study. J Clin Oncol 2015;33:3938–44.
5. Roka S, Rudas M, Taucher S, Dubsky P, Bachleitner-Hofmann T, Kandioler D, et al. High nuclear grade and negative estrogen receptor are significant risk factors for recurrence in DCIS. Eur J Surg Oncol 2004;30:243–7.
6. Ringberg A, Anagnostaki L, Anderson H, Idvall I, Ferno M, South Sweden Breast Cancer Group. Cell biological factors in ductal carcinoma *in situ* (DCIS) of the breast-relationship to ipsilateral local recurrence and histopathological characteristics. Eur J Cancer 2001;37:1514–22.
7. Kerlikowske K, Molinaro AM, Gauthier ML, Berman HK, Waldman F, Bennington J, et al. Biomarker expression and risk of subsequent tumors after initial ductal carcinoma *in situ* diagnosis. J Natl Cancer Inst 2010;102:627–37.
8. Provenzano E, Hopper JL, Giles GG, Marr G, Venter DJ, Armes JE. Biological markers that predict clinical recurrence in ductal carcinoma *in situ* of the breast. Eur J Cancer 2003;39:622–30.
9. Kuerer HM, Lari SA, Arun BK, Hu CY, Brewster A, Mittendorf EA, et al. Biologic features and prognosis of ductal carcinoma *in situ* are not adversely impacted by initial large body mass. Breast Cancer Res Treat 2012;133:1131–41.
10. Allred DC, Anderson SJ, Paik S, Wickerham DL, Nagtegaal ID, Swain SM, et al. Adjuvant tamoxifen reduces subsequent breast cancer in women with estrogen receptor-positive ductal carcinoma *in situ*: a study based on NSABP protocol B-24. J Clin Oncol 2012;30:1268–73.
11. Claus EB, Chu P, Howe CL, Davison TL, Stern DF, Carter D, et al. Pathobiologic findings in DCIS of the breast: morphologic features, angiogenesis, HER-2/neu and hormone receptors. Exp Mol Pathol 2001;70:303–16.
12. Cuzick J, Sestak I, Pinder SE, Ellis IO, Forsyth S, Bundred NJ, et al. Effect of tamoxifen and radiotherapy in women with locally excised ductal carcinoma *in situ*: long-term results from the UK/ANZ DCIS trial. Lancet Oncol 2011; 12:21–9.
13. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer 2005;93:387–91.
14. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol 2010;28:2784–95.
15. Press M, Spaulding B, Groshen S, Kaminsky D, Hagerty M, Sherman L, et al. Comparison of different antibodies for detection of progesterone receptor in breast cancer. Steroids 2002;67:799–813.
16. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998;11:155–68.
17. Detre S, Jotti GS, Dowsett M. A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. J Clin Pathol 1995;48:876–8.
18. Pinder SE, Duggan C, Ellis IO, Cuzick J, Forbes JF, Bishop H, et al. A new pathological system for grading DCIS with improved prediction of local recurrence: results from the UKCCCR/ANZ DCIS trial. Br J Cancer 2010;103: 94–100.
19. National Pathology Co-ordinating Group. Pathology reporting of breast disease. Sheffield, England: NHSBSP Publication. NHS Cancer Screening Programmes and The Royal College of Pathologists; 2005. p. 50–9.
20. Mark SD, Katki HA. Specifying and implementing nonparametric and semiparametric survival estimators in two-stage (nested) cohort studies with missing case data. J Am Statist Assoc 2006;101:460–71.
21. Katki HA, Mark SD. Survival analysis for cohorts with missing covariate information. The R Journal 2008;8:14–9.
22. Wang SY, Shamliyan T, Virnig BA, Kane R. Tumor characteristics as predictors of local recurrence after treatment of ductal carcinoma *in situ*: a meta-analysis. Breast Cancer Res Treat 2011;127:1–14.
23. Zhang X, Dai H, Liu B, Song F, Chen K. Predictors for local invasive recurrence of ductal carcinoma *in situ* of the breast: a meta-analysis. Eur J Cancer Prev 2016;25: 19–28.
24. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883–92.
25. de Bruin EC, McGranahan N, Mitter R, Salm M, Wedge DC, Yates L, et al. Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. Science 2014;346:251–6.
26. Gatenby RA, Silva AS, Gillies RJ, Frieden BR. Adaptive therapy. Cancer Res 2009; 69:4894–903.
27. Silverstein MJ, Lagios MD. Treatment selection for patients with ductal carcinoma in situ (DCIS) of the breast using the University of Southern California/ Van Nuys (USC/VNPI) prognostic index. Breast J 2015;21:127–32.
28. Curigliano G, Disalvatore D, Esposito A, Pruneri G, Lazzeroni M, Guerrieri-Gonzaga A, et al. Risk of subsequent in situ and invasive breast cancer in human epidermal growth factor receptor 2-positive ductal carcinoma *in situ*. Ann Oncol 2015;26:682–7.
29. Thompson AM, Clements K, Cheung S, Pinder SE, Lawrence G, Sawyer E, et al. Management and 5-year outcomes in 9938 women with screen-detected ductal carcinoma *in situ*: the UK Sloane Project. Eur J Cancer 2018;101:210–9.