ARTICLE

Macrodissection prior to closed system RT-qPCR is not necessary for estrogen receptor and HER2 concordance with IHC/FISH in breast cancer

Swati Gupta[1] · Navin R. Mani[1] · Daniel E. Carvajal-Hausdorf[1,2] · Veerle Bossuyt[1] · Kenneth Ho[3] · Jodi Weidler[4] ·
Wendy Wong[3] · Brian Rhees[3] · Michael Bates[4] · David L. Rimm[1]

Abstract

An on-demand, closed RT-qPCR, the GeneXpert (GX) system, has the potential to provide biomarker information in low-resourced settings and elsewhere. We used this system with a research use only version of the Breast Cancer STRAT4 cartridge that measures the mRNA expression levels of *ERBB2*, *ESR1*, *PGR*, and *MKi67*. Here we evaluated the impact of non-macrodissected (non m-d) versus macrodissected (m-d) samples using STRAT4 on formalin-fixed, paraffin-embedded (FFPE) core needle biopsies. Two cohorts were assessed: (1) 60 FFPE infiltrating ductal carcinoma (IDCA) cases and (2) 20 FFPE IDCA cases with ductal carcinoma in situ (DCIS) with a range of HER2 expression as determined by clinical immunohistochemistry and fluorescence in situ hybridization (IHC/FISH). We observed about half of the core needle biopsy area as invasive tumor in both IDCA (mean = 51.5%) and IDCA with DCIS (mean = 53.5%) cohorts, but also found the mRNA levels were independent of tumor area. We found excellent agreement of the mRNA transcript level between the paired samples, m-d versus non m-d, for *ERBB2*, *ESR1*, *PGR*, and *MKi67* for both the IDCA and IDCA with DCIS cohorts. No significant difference ($P > 0.99$) was observed when we compared the mRNA transcript level between the paired samples m-d versus non m-d. In addition, we noted a significant concordance ($P < 0.001$) between RT-qPCR and IHC/FISH for HER2-positivity, ER-positivity, and PR-positivity, independent of specimen dissection. These data suggest that mRNA expression for *ERBB2*, *ESR*, and *PGR* is sufficiently low in surrounding tissue cells such that macrodissection is not required for assessment of key breast cancer mRNA markers and is independent of the amount of input tumor. This approach may be valuable in settings lacking pathology expertise or using specimen types, such as fine-needle aspirates, where it may be challenging to separate non-tumor from tumor tissue.

✉ David L. Rimm
david.rimm@yale.edu

[1] Department of Pathology, Yale University School of Medicine, New Haven, CT, USA

[2] Anatomía Patológica, Clínica Alemana—Facultad de Medicina Universidad del Desarrollo, Santiago, Chile

[3] Division of Oncology Research and Development, Oncology, Cepheid, Sunnyvale, CA, USA

[4] Medical and Scientific Affairs and Strategy, Oncology, Cepheid, Sunnyvale, CA, USA

Introduction

Breast cancer is one of the most common cancers in women worldwide in both developing and developed countries [1, 2]. Worldwide, newly diagnosed breast cancer cases are estimated at ~1.7 million annually, with the highest numbers observed in Europe (30%) and the US (12%) [2]. Over the years, breast cancer mortality in the developed world has decreased by 36% due to improved treatment and early detection [3]. Overexpression of human epidermal growth factor (HER2/*ERBB2*) at the gene and protein level is a standard of care in the US and Europe for breast cancer patients since it is predictive of response to HER2-targeted therapies [4, 5]. Estrogen receptor (ESR/*ESR1*) and progesterone receptor (PGR/*PGR*) are prognostic factors for aggressive disease and predict response to tamoxifen and other hormonal therapies [6–9].
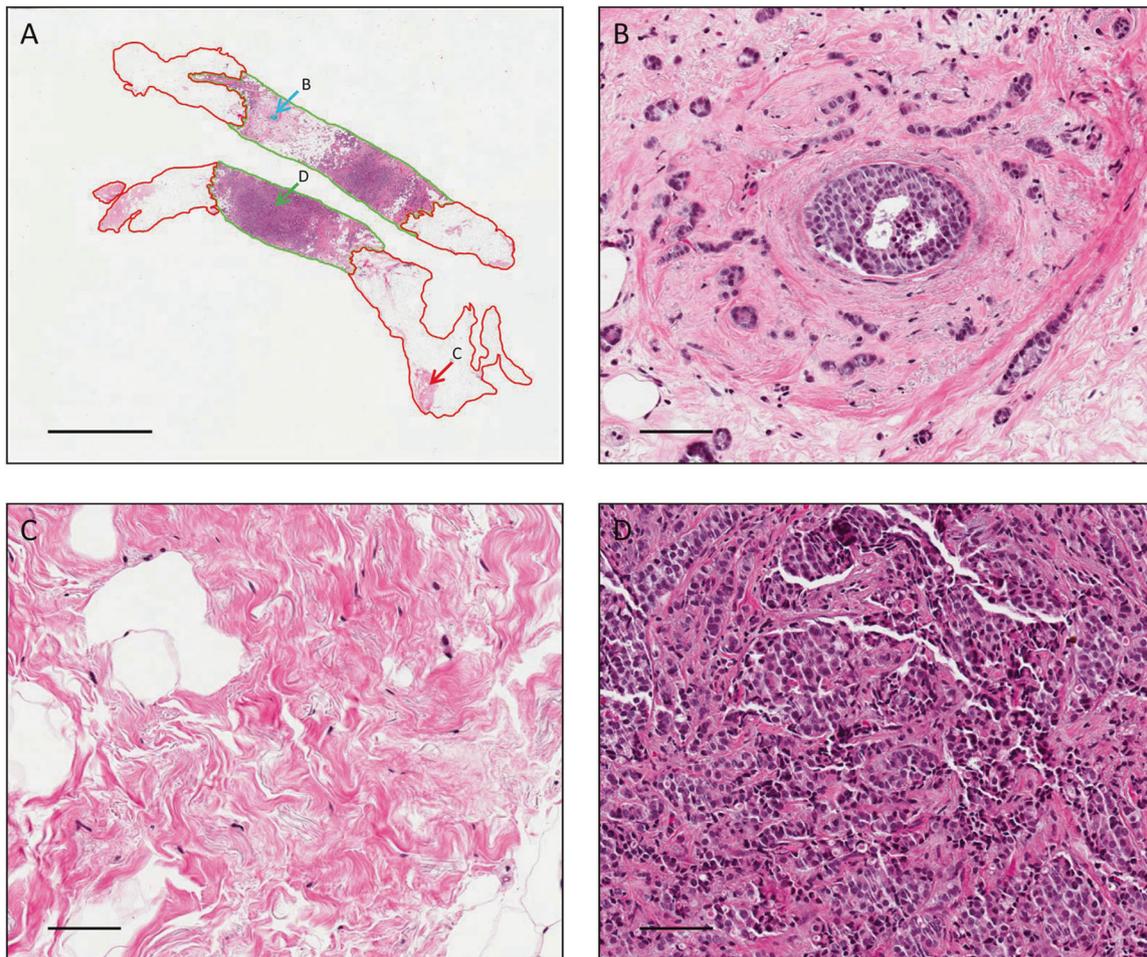
Fig. 1 Representative images of H&E stained IDCA with DCIS breast cancer section showing overall view of the core needle biopsy (**a**), DCIS tumor area (**b**), non-tumor tissue area (**c**), and tumor tissue area (**d**). Magnification panels and scale bar shown at (**a**) are ×0.6 and 4 mm and **b**–**d** are ×20 and 100 um. Green represents tumor area, red represents stroma area, and cyan represents DCIS area

The measurement of HER2, ER, PR, and sometimes Ki-67 protein expression by immunohistochemistry (IHC) represents the standard of care in the clinic. In addition, *ERBB2* copy number status is often assessed by fluorescence in situ hybridization (FISH) when HER2 IHC results are equivocal [10]. While mRNA is used to assess these same markers as part of the commonly used Oncotype Dx test, mRNA is not commonly used as the basis for therapeutic regimen selection, and is not endorsed in the ASCO/CAP guidelines [11]. One reason for this is that the in situ value of IHC tests is thought to prevent the false positive tests that might be seen due to contaminating mRNA expression for these key markers in non-tumor tissue.

Here we determine whether mRNA expression for key breast cancer markers, *ERBB2*, *ESR1*, *PGR*, and *MKi67*, is equivalent in non-macrodissected sections (non m-d) compared with tumor-specific macrodissected sections (m-d) using core needle biopsies with infiltrating ductal carcinoma (IDCA) and IDCA with ductal carcinoma in situ (DCIS) using a real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay (GeneXpert® Breast Cancer STRAT4 RUO Assay, Cepheid, Sunnyvale, CA, USA). Additionally, we assessed the correlation between RT-qPCR and clinically determined IHC/FISH for HER2-positivity, ER-positivity, and PgR-positivity in the paired samples, non m-d versus m-d.

Materials and methods

Patient and tissues

Breast carcinoma patient cohorts were selected by screening the tumor bank of Yale University for 60 formalin-fixed, paraffin-embedded (FFPE) IDCA and 20 FFPE IDCA with DCIS diagnosed between 2012 and 2015. The results for CLIA-certified HER2 IHC/FISH scoring according to the American Society of Clinical Oncology, and the College of American Pathologists (ASCO/CAP) guidelines were extracted from the pathology reports. The tissues were collected under IRB protocol ID 9505008219.

Table 1 Percentage epithelial tumor area in the IDCA cohort

% Tumor epithelium, HER2 0+, $n = 13$	
Mean	49.5
Median	39
SD	26.2
Minimum	22
Maximum	100
% Tumor epithelium, HER2 1+, $n = 17$	
Mean	57.2
Median	58
SD	21.3
Minimum	25
Maximum	94
% Tumor epithelium, HER2 2+, $n = 16$	
Mean	55.5
Median	60
SD	21.6
Minimum	13
Maximum	85
% Tumor epithelium, HER2 3+, $n = 14$	
Mean	42
Median	41
SD	22.7
Minimum	14
Maximum	85

Table 2 Percentage epithelial tumor and DCIS area in the IDCA with DCIS cohort

	% Tumor epithelium	% DCIS
HER2 0+, $n = 5$		
Mean	46.8	0.6
Median	28	0.4
SD	29.9	0.6
Minimum	22	0.1
Maximum	81	1.5
HER2 1+, $n = 8$		
Mean	55.3	1.2
Median	55.5	0.7
SD	18.1	1.2
Minimum	20	0.1
Maximum	77	3.9
HER2 2+, $n = 7$		
Mean	58.6	1.7
Median	52	0.9
SD	24.5	2.0
Minimum	30	0.1
Maximum	97	5.4

Area measurement

Samples were sectioned at the Yale pathology tissue service. Imaging of hematoxylin and eosin sections was completed on Aperio ScanScope Console (v10.2.0.2352). Tumor, stroma, and DCIS areas circled by a pathologist were measured using pen and annotation tool of Aperio ImageScope (v12.3.2.8013).

GeneXpert Breast Cancer STRAT4 assay

The BC STRAT4 assay was performed as previously described [12]. Briefly, 5-µM-thick FFPE tissue sections were collected either as a whole tissue section, non m-d, or macrodissected, m-d, to collect tumor only. Samples were lysed using 5 µl Proteinase K and 260 µl FFPE lysis reagent and incubated for 30 min at 80 °C. Then, 260 µL of ≥95% ethanol was added to the lysed samples and vortexed to mix. The mixture was transferred to a BC STRAT4 cartridge and tested on the GeneXpert (GX) system. This assay isolates the total RNA, performs a 1-step RT-PCR and provides Ct values for the endogenous control, CYFIP, and the target genes, *ERBB2*, *ESR1*, *PGR*, and *MKi67*. Results were expressed as a dCt value, defined as the Ct of the control gene (CYFIP1) minus the Ct of each of the target genes (*ERBB2*, *ESR1*, *PGR*, or *MKi67*). The BC STRAT4 dCt cutoffs for *ERBB2*, *ESR*, *PGR*, and *MKi67* were established in a breast cancer patient cohort, based on the highest concordance achieved between relative RNA amplification and corresponding protein expression by IHC as the gold standard [13].

Statistical analysis

All datasets were analyzed and plotted using GraphPad Prism v7.0 software for Windows (GraphPad Software, Inc., La Jolla, CA). The concordance between non m-d and m-d groups was assessed using linear regression coefficient (R^2). The comparison amongst non m-d and m-d groups was performed using two-tailed unpaired Student's t-tests. Fisher's exact test two-sided was used to compare the RT-qPCR and IHC results for HER2-positivity, ER-positivity, and PgR-positivity. All P-values under 0.05 were considered statistically significant.

Results

Tumor epithelium and DCIS area in cohorts

Tumor epithelium and DCIS were circled and the percentage of tumor area was measured to assess the stroma and DCIS components present in all breast cancer samples (Fig. 1). The distribution of percentage tumor epithelium
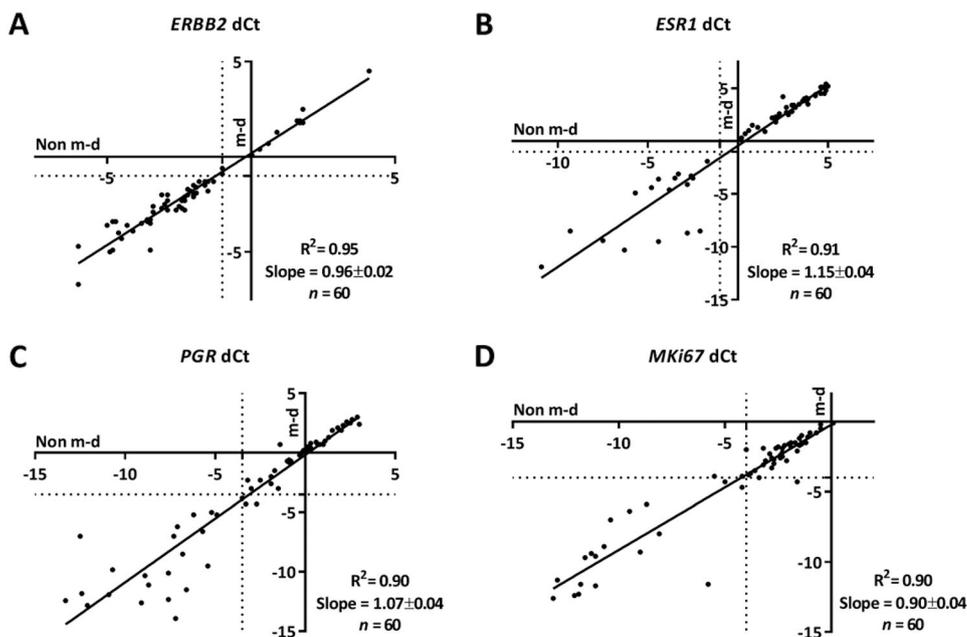
Fig. 2 Correlation between non m-d versus m-d samples for RT-qPCR in IDCA cohort. Regression of mRNA quantification for *ERBB2* (**a**), *ESR1* (**b**), *PGR* (**c**), and *MKi67* (**d**). The respective R^2 and slope values are indicated in the chart. Non m-d not macrodissected, m-d macrodissected

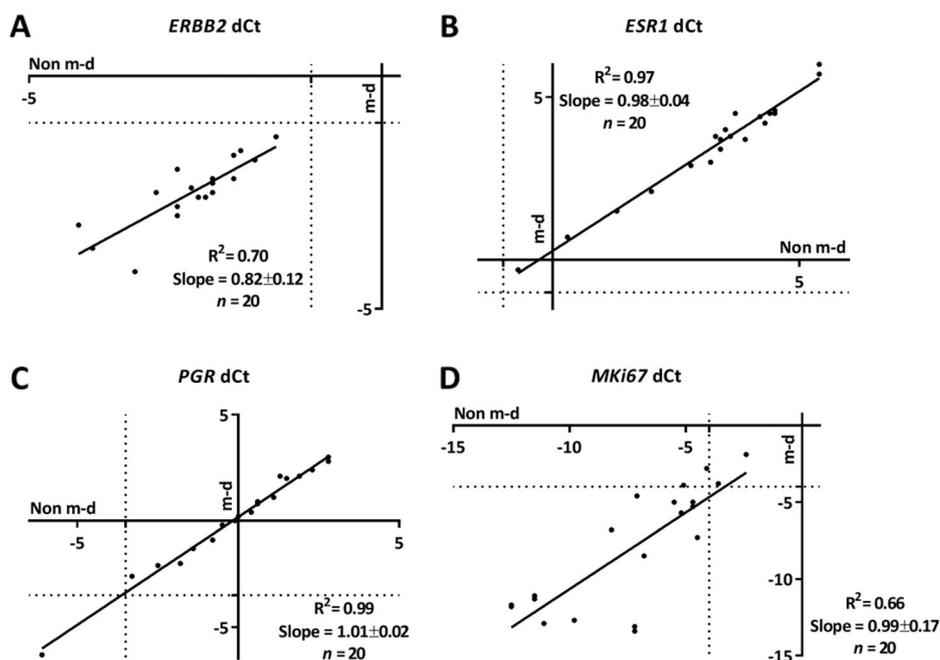**Fig. 3** Correlation between non m-d versus m-d samples for RT-qPCR in IDCA with DCIS cohort. Regression of mRNA quantification for *ERBB2* (**a**), *ESR1* (**b**), *PGR* (**c**), and *MKi67* (**d**). The respective R^2 and slope values are indicated in the chart. Non m-d not macrodissected, m-d macrodissected

area in the IDCA cohort are shown in Table 1. Similarly, the distributions of percentage tumor epithelium and percentage DCIS area in the IDCA cohort is shown in Table 2. We observed about half of the core needle biopsy area as stroma in both the IDCA (mean percentage tumor = 51.5%) and IDCA with DCIS (mean percentage tumor = 53.5%) cohorts. The percentage tumor epithelium in this small cohort ranges from 13 to 100%. In addition, we found a range of DCIS positivity of 0.1–5.4% in the IDCA with DCIS cohort.

### Breast cancer mRNA markers assessed as a function of macrodissection

To evaluate the impact of macrodissection, we measured mRNA expression of key breast cancer genes, *ERBB2*, *ESR1*, *PGR*, and *MKi67*, between the paired samples non m-d versus m-d from 60 FFPE IDCA cases and 20 FFPE IDCA with DCIS cases with a range of HER2 expression determined by HER2 IHC/FISH. We saw excellent agreement of the resulting dCt values, between the paired

Table 3 Concordance between RT-qPCR and IHC/FISH for HER2 in IDCA cohort

	HER2 positive	HER2 negative	Total
Non m-d samples ($P < 0.001$; sensitivity $= 0.86$; specificity $= 1$; PPV $= 1$; NPV $= 0.96$)			
dCt ≥ -1	12	0	12
dCt < -1	2	46	48
Total	14	46	60
m-d samples ($P < 0.001$; sensitivity $= 0.86$; specificity $= 1$; PPV $= 1$; NPV $= 0.96$)			
dCt ≥ -1	12	0	12
dCt < -1	2	46	48
Total	14	46	60

Table 4 Concordance between RT-qPCR and IHC/FISH for ESR in IDCA cohort

	ER positive	ER negative	Total
Non m-d samples ($P < 0.001$; sensitivity $= 0.98$; specificity $= 1$; PPV $= 1$; NPV $= 0.94$)			
dCt ≥ -1	43	0	43
dCt < -1	1	16	17
Total	44	16	60
m-d samples ($P < 0.001$; sensitivity $= 0.98$; specificity $= 1$; PPV $= 1$; NPV $= 0.94$)			
dCt ≥ -1	43	0	43
dCt < -1	1	16	17
Total	44	16	60

Fig. 4 Comparison between pathologists determined IHC/FISH and RT-qPCR for non m-d versus m-d samples in IDCA cohort. Dot plot showing mRNA quantification of *ERBB2* (**a**), *ESR1* (**b**), *PGR* (**c**), and *MKi67* (**d**) determined to be 0+, 1+, 2+, and 3+ by IHC and confirmed by FISH. Non m-d not macrodissected, m-d: macrodissected
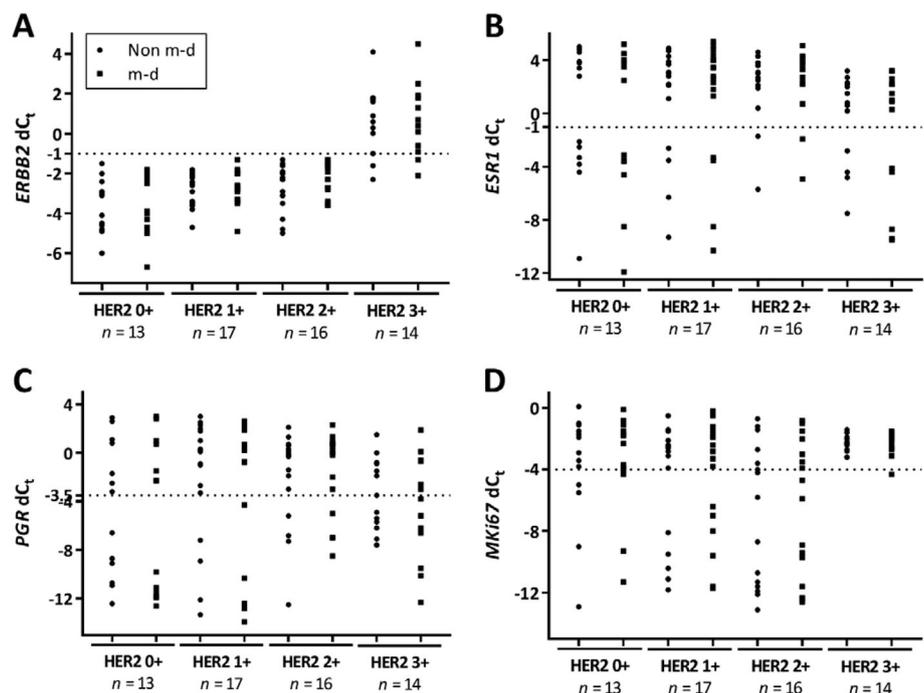
samples, m-d versus non m-d, for *ERBB2* ($R^2 = 0.95$), *ESR1* ($R^2 = 0.91$), *PGR* ($R^2 = 0.90$), and *MKi67* ($R^2 = 0.90$) in the IDCA cohort (Fig. 2). Since contaminating DCIS in IDCA specimens could potentially contribute *ERBB2* mRNA signal to in vitro assays, a second cohort was assessed where DCIS accompanied IDCA in every case. Here high concordance of the resulting dCt between the paired samples, m-d versus non m-d, was observed for *ERBB2* ($R^2 = 0.70$), *ESR1* ($R^2 = 0.97$), *PGR* ($R^2 = 0.99$), and *MKi67* ($R^2 = 0.66$) (Fig. 3). Experiments were performed in triplicates for cases closer to the *ERBB2* threshold ($-0.5 < dCt < -1.5$) and the agreement between non m-d versus m-d samples for every case was observed (Supplemental Fig. 1). When clinical cutoffs for the various analytes are applied, the concordance remains strong, no specimen in either cohort changed from

positive to negative or vice versa as a function of non m-d or m-d for *ERBB2* and *ESR* (Tables 3 and 4). In addition, no significant difference ($P > 0.99$) was observed when we compared the dCt between the paired samples, m-d versus non m-d, for *ERBB2*, *ESR1*, *PGR*, and *MKi67* in the IDCA cohort (Fig. 4) and IDCA with DCIS cohort (Fig. 5).

Concordance between RT-qPCR and IHC/FISH measurements

To assess the concordance agreement between RT-qPCR and IHC/FISH classification, we used the predefined BC STRAT4 dCt cutoffs for *ERBB2*, *ESR*, and *PGR* positivity and compared those to clinical IHC/FISH results in the IDCA cohort [12, 13]. We found a significant concordance

Fig. 5 Comparison between pathologist determined IHC/FISH and RT-qPCR for non m-d versus m-d samples in IDCA with DCIS cohort. Dot plot showing mRNA quantification of *ERBB2* (**a**), *ESR1* (**b**), *PGR* (**c**), and *MKi67* (**d**) determined to be 0+, 1+, and 2+ by IHC and confirmed by FISH. Non m-d not macrodissected, m-d macrodissected

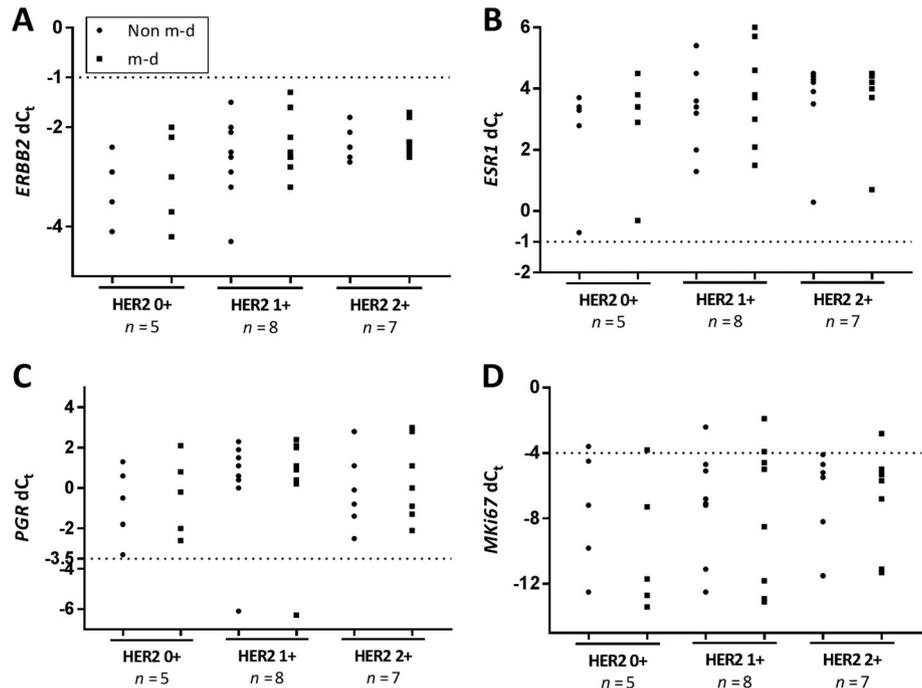**Table 5** Concordance between RT-qPCR and IHC/FISH for PGR in IDCA cohort

	PR positive	PR negative	Total
Non m-d samples ($P < 0.001$; sensitivity = 0.86; specificity = 0.88; PPV = 0.95; NPV = 0.71)			
dCt ≥ −3.5	37	2	39
dCt < −3.5	6	15	21
Total	43	17	60
m-d samples ($P < 0.001$; sensitivity = 0.84; specificity = 1; PPV = 1; NPV = 0.71)			
dCt ≥ −3.5	36	0	36
dCt < −3.5	7	17	24
Total	43	17	60

between RT-qPCR and IHC/FISH for HER2-positivity for the paired samples, non m-d and m-d (95.0%; $P < 0.001$; sensitivity = 0.86; specificity = 1; PPV = 1; NPV = 0.96) (Table 3), ESR-positivity for the paired samples, non m-d and m-d (98.3%; $P < 0.001$; sensitivity = 0.98; specificity = 1; PPV = 1; NPV = 0.94) (Table 4) and PGR-positivity for the paired samples, non m-d (86.7%; $P < 0.001$; sensitivity = 0.86; specificity = 0.88; PPV = 1; NPV = 0.71) versus m-d (88.3%; $P < 0.001$; sensitivity = 0.84; specificity = 1; PPV = 1; NPV = 0.71) (Table 5), respectively.

RT-qPCR dCt scores were independent of tumor epithelium and DCIS area

We assessed the area-specific distribution of mRNA expression in both the IDCA and IDCA with DCIS cohorts.

The mRNA transcript levels of *ERBB2*, *ESR1*, *PGR*, and *MKi67* were independent of the percent epithelial tumor area present in the IDCA and IDCA with DCIS cohorts (Fig. 6a, b and data not shown). Furthermore, the mRNA transcript levels of *ERBB2*, *ESR1*, *PGR*, and *MKi67* were also independent of the percent DCIS area present in IDCA with DCIS cohort (Fig. 6c, d and data not shown).

Discussion

Breast cancer tissues show both architectural and cellular heterogeneity, representing a blend of tumor cells, healthy epithelial ducts, surrounding stroma and, sometimes DCIS cells. Therefore, RNA or protein in vitro extracts from whole tissue lysates also represent the presence of a variety of cell populations in the tissue under examination and potentially limits the ability to identify tumor cell-specific signals. As a consequence, several methods have been reported for tissue microdissection, including gross dissection of frozen tissue blocks [14], irradiation of manually ink-stained sections [15], touch preparations of frozen tissue specimens [16], macrodissection with manual tools [17–19], and laser capture microdissection [20]. The simplest of these methods is manual tool macrodissection of the FFPE slide. This has been the method adopted and is in routine use for the Oncotype Dx test, MammaTyper assay, and possibly other RNA-based tests. Recently, Laible et al. [21] showed that microdissection of FFPE tissue sections did not appear to be necessary for the MammaTyper assay, which assigns breast cancer specimens into the molecular subtypes according to the mRNA

Fig. 6 Area-specific distribution of mRNA expression in IDCA and IDCA with DCIS cohort. Scatterplots depicting distribution of the percentage epithelial tumor area (**a**, **b**) and percentage DCIS area (**c**, **d**) versus mRNA quantification for *ERBB2* (**a**, **c**) and *ESR1* (**b**, **d**) in IDCA and IDCA with DCIS cohort
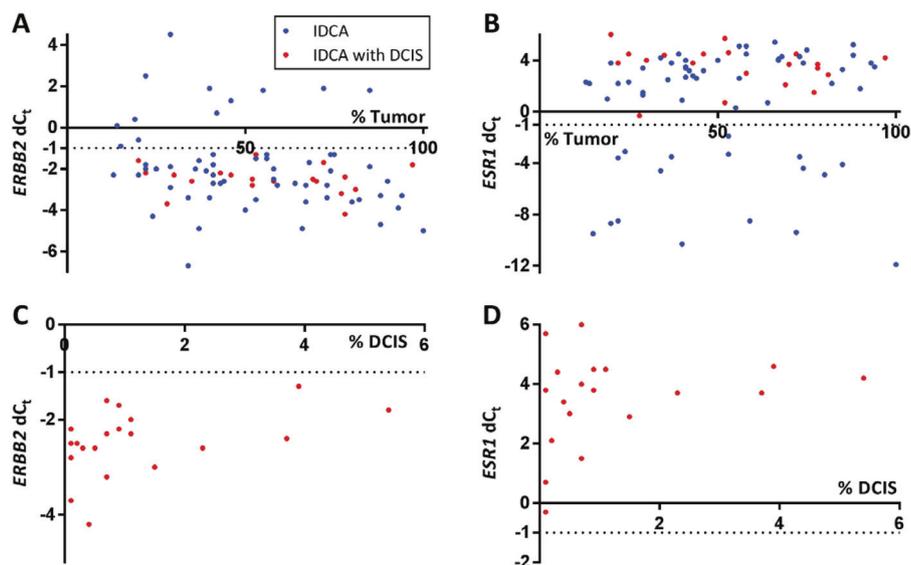
expression of *ERBB2*, *ESR1*, *PGR*, and *MKi67*. Our results with STRAT4 supports this study as we found no differences between results obtained using macrodissection and whole sections for the same targets. A few cases show discordance between RT-QPCR and IHC/FISH. This could be due to variance inherent in assays or actual discordance due to biological variance between RNA levels and protein levels, since not all RNA is translated. Similarly, not all cases with FISH amplification show increased protein and this may be a similar biological phenomenon.

IHC is the current practice for the measurements of HER2, ER, PR, and Ki-67 protein expression in breast cancer and *ERBB2* status is assessed by FISH when HER2 IHC results are equivocal [10]. Over the years, the measurement of RNA for breast cancer markers has emerged using several commercially available platforms [22–24], most significantly for multiparameter prognostic testing. However, simple qRT-PCR has failed to gain acceptance for HER2 assessment. Here we showed a significant concordance ($P < 0.001$) between the GX closed system RT-qPCR assay and IHC/FISH scoring for HER2, ER, and PR for the paired samples, non m-d versus m-d. These results suggest that the closed system RT-qPCR assay may have potential to be utilized in parallel to current practice for the measurements of HER2, ER, and PR.

There are a number of limitations to this work. Perhaps the most significant is that it is underpowered to assess concordance with IHC. While we show concordance here, the main focus of this work is the comparison with m-d vs non m-d. Other larger studies are underway to assess concordance between IHC and RT-PCR. Since mixed population of invasive carcinoma and DCIS can create discrepancies in biomarker expression, future studies might address this issue by focusing on cases with HER2

overexpression in DCIS, but not in an adjacent invasive component. Also, since this work focused on HER2, we did not assess cases with small foci of ER/PR-negative invasive carcinoma that have significant amounts of benign glandular tissue. This could contribute to false positive results and may be addressed in future studies. Another limitation of this work is the focus on core needle biopsies. While our intention was only to assess small specimens (core biopsies), in the future we will attempt to assess FNA and whole, full-sized resection specimens. The conclusions of this work cannot necessarily be applied to other specimen types.

Conclusions

In summary, we compared mRNA expression for key breast cancer markers in non m-d versus m-d in IDCA and IDCA with DCIS cohorts and found that m-d of whole sections is not required for accurate assessment of these genes in a closed system RT-qPCR assay. The simplicity of the assay workflow may be particularly valuable in low-resourced settings where routine access to pathology expertise and to high-quality IHC/FISH is challenging.

Compliance with ethical standards

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