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Microarray and RNA *in situ* hybridization assay for recurrence risk markers of breast carcinoma and ductal carcinoma *in situ*: evidence supporting the use of diverse pathways panels

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Abstract

Background: Breast tumor stratification by recurrence-risk is a critical for deciding patient treatment. Here an approach combining cancer pathways microarray data complemented by RNA *in situ* hybridization (ISH) was investigated as a means for recurrence marker discovery and visualization in pathology specimens.

Methods: LncRNA and mRNA expressions in breast carcinomas with low (n=8) versus intermediate/high (n=10) recurrence-scores as estimated by 21-gene assay and pathology review were compared by microarray assay. Tissue microarrays were prepared from breast carcinomas (n=20) and ductal carcinoma *in situ* (DCIS) specimens (n=84 patients) with known outcomes. Thirteen RNA ISH assays were performed: lncRNAs (*BBC3-1*, *FER3*, *RAD21-AS1*, *ZEB1-2*) and mRNAs (*GLO1*, *GLTSCR2*, *TGFB1*, *TLR2*) [implicated by the microarray data]; *MKI67*; a pooled panel of recurrence-associated proliferation markers (*BIRC5*, *Cyclin B1*, *MKI67*, *MYBL2*, *STK15*); a pooled panel of non-proliferation recurrence-associated markers (*CEACAM5*, *HTF9C*, *NDRG1*, *TP53*, *SLC7A5*); and lncRNAs *H19* and *HOTAIR*.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

Results: Seven lncRNAs and 10 mRNAs showed significantly ($P<0.05$) altered up or down-regulation by microarray assay: carcinoma RNA ISH staining did not mirror these patterns. *HOTAIR* staining was associated with a higher breast cancer recurrence score ($P=0.0152$); qualitatively, *H19* was massively expressed in a metaplastic triple negative breast carcinoma. Among the DCIS cohort, significant associations with multiple outcome variables were noted for *TGFB1* and the non-proliferation panel (P -value range: 0.0001 to 0.047); proliferation panel staining showed an association with increasing DCIS grade ($P=0.0269$) but not with outcomes.

Conclusions: The findings support recurrence-risk estimation by the use of multi-marker panels that are representative of diverse cellular pathways rather than over-reliance on proliferation targets. *H19*, *HOTAIR* and *TGFB1* RNA ISH show potential for selective diagnostics.

Keywords

Breast Cancer; DCIS; Recurrence; Microarray Analysis; In Situ Hybridization; lncRNA; mRNA

INTRODUCTION

Stratification of breast cancer by risk for recurrence is important for deciding patient management and treatment. Available predictive tests include the Agendia Mammoprint (microarray), Breast Cancer Index (qRT-PCR), EndoPredict (qRT-PCR), Oncotype DX (qRT-PCR), and Prosigna (NanoString technology) assays [1–5]. Immunohistochemistry (IHC) tests have also been developed such as IHC4 and Mammostrat that screen for four or five different targets respectively and estimate risk using stain scoring algorithms [6, 7]. IHC is an attractive option because of the possibility of onsite testing and pathologists' familiarity with the assay, whereas molecular tests typically require sending patient specimens to a reference laboratory. IHC is routinely used for a wide range of pathology diagnoses; however, there have been difficulties validating and standardizing IHC for reasons such as alternative antibody clone choices, epitope retrieval methodologies, and IHC scoring approaches [8]. RNA *in situ* hybridization (ISH) has been proposed as an alternative to IHC because of its target sensitivity and specificity, clone and epitope retrieval technique independence, and adaptability for use on autostainers [9].

In this exploratory study, a microarray assay was performed comparing long non-coding RNA (lncRNA) and mRNA expression among breast cancer samples with Oncotype DX (Genomic Health, Inc., Redwood City, CA) 21-gene assay recurrence scores (RS) used as a surrogate for 10 year outcome. Implicated differentially expressed lncRNA or mRNA markers were then investigated by RNA ISH assay of breast carcinoma and DCIS tissue microarrays (TMAs). The TMAs were also screened by RNA ISH for *MKI67*, a pooled proliferation gene panel (adapted from the Oncotype DX assay), a pooled non-proliferation gene panel (adapted from the Mammostrat IHC test), and for lncRNAs *H19* and *HOTAIR* previously identified as having potential RNA ISH utility [11]. The study aims were to identify novel risk markers by microarray data analyses and to examine their translatability as differentially expressed staining patterns detectable by RNA ISH in breast tumor tissues; proliferative vs. non-proliferative pathway markers were also examined by RNA ISH.

MATERIALS AND METHODS

All investigations were performed with University of Vermont (UVM) institutional review boards' approvals.

Patients:

To limit cohort heterogeneity, all breast cancer patients were post-menopausal with hormone positive, HER2 negative (0/negative, 1+ and 2+ with non-amplified ratio by ISH) non-metastatic tumors; two triple negative breast cancers (TNBC) were included for contrast purposes (Table 1). Specimens were selected by CoPath software (Sunquest Information Systems, Tucson, AZ) search of UVM Medical Center pathology archives. All specimens (n=20) were from 2012–2014 (with 5-year available follow-up). During this time period, Oncotype DX recurrence score (RS) was classed as Low Risk (RS <18), Intermediate Risk (RS 18–30) or High Risk (RS ≥ 31) [4]. There is an absence of patients receiving high RS values at our institute as tumors with aggressive clinical features are treated accordingly without referral for a RS test. H&E slides of formalin-fixed, paraffin-embedded (FFPE) tissue blocks were obtained for all 20 patients and reviewed to select blocks with high tumor content for the preparation of samples for microarray assay and TMA construction.

TMAs of breast ductal carcinoma *in situ* (DCIS) specimens from a prior study [10] were also included in this study and comprised samples (1996–2008) collected from 84 patients (mean age 58 years; range 35–82). The cohort included 55 instances of recurrences (33 ipsilateral, 18 contralateral, 4 bilateral) with time recurrences in the range 6–188 months; 24 patients recurred within 5 years of follow-up. Treatment histories included 26 radiation therapy, 11 hormone therapy, 20 total mastectomy, 28 no treatment (surgical excision, no adjuvant treatment), 6 unknown (Table 2).

Microarray Analysis

To ensure sufficient RNA recovery for microarray assays of FFPE samples, three 2 mm diameter ~3 mm depth core punches per patient were sampled from tumor tissue block tumor regions using an H&E stained section to guide tumor block punching. The FFPE tissue cores were shipped to Arraystar Inc., Rockville, MD for RNA extractions and microarray assay (Human Cancer Pathway Microarray that screens for 2,829 lncRNAs and 1,906 mRNAs) and statistical analyses according to their optimized protocols. Briefly, lncRNAs/mRNAs were purified from total RNA after removal of rRNA (Arraystar rRNA removal kit). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method. The labeled cRNAs were purified by RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured by NanoDrop ND-1000. 1 μg of each labeled cRNA was fragmented by adding 5 μl 10 × Blocking Agent and 1 μl of 25 × Fragmentation Buffer, then heated the mixture at 60 °C for 30 min, finally 25 μl 2 × GE Hybridization buffer was added to dilute the labeled cRNA. 50 μl of hybridization solution was dispensed into the gasket slide and assembled to the lncRNA expression microarray slide. The slides were incubated for 17 hours at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed and scanned using the Agilent Scanner G2505C. Raw

data collection for each tumor was by importing scanned images into Agilent Feature Extraction software for raw data extraction. Expression profiling of data was by quantile normalization of raw data and subsequent data processing performed using the R limma software package. After quantile normalization of the raw data, low intensity filtering was performed, and the lncRNAs/Coding Genes with at least 1 of the samples having flags in “P” or “M” (“All Targets Value”) were retained for further analyses allowing comparison of lncRNA and mRNA expression among low RS vs. intermediate/high RS specimens.

Tissue Microarray (TMA) Preparation

Patient specimen H&E slides were reviewed to identify cancer rich FFPE tissue blocks from the patients shown in Table 1. Fresh H&E slides were prepared from selected specimens. Tumor rich areas were ink-marked on the slides to use as reference marks for sampling 2.0 mm core punches for TMA prep; one normal and two tumor regions were core sampled per patient. Five micron sections were slide mounted for H&E and RNA ISH procedures.

RNA *In Situ* Hybridization

Manual RNAscope ISH (Advanced Cell Diagnostics, Inc., Newark, CA) was performed using a HybEz hybridization oven. TMA assay conditions were optimized using positive control probes (*UBC*, *PPIB*, *Polr2A* [for high (>20), medium (10–20) and low (3–15) target copies per cell detection respectively]) and negative control probe (*B. subtilis DapB*) to confirm an absence of signals.

Further to microarray acquired data (Tables 3A and 3B), in the absence of any prior literature citations four lncRNAs (*BBC3*, *FER3*, *RAD21*, *ZEB1*) were chosen at random and four mRNAs (*GLO1*, *GLTSCR2*, *TGFB1*, *TLR2*) selected after literature review were selected for ISH probes. TMAs were also screened with an *MKI67* probe and a pooled panel of the five proliferation markers: *BIRC5* (*Survivin*), *Cyclin B1* (*CCNB1*), *MKI67*, *MYBL2*, *STK15* (*AURKA*) that are components of the Oncotype DX breast cancer 21-gene recurrence assay (16 cancer genes and 5 reference genes); the same 5 proliferation markers are also part of the Oncotype DX Breast DCIS score assay that evaluates a total 7 cancer genes and 5 reference genes [10]. A panel of the five Mammostrat targets (*SLC7A5*, *p53*, *NDRG1*, *HTF9C*, *CEACAM5*) that are not overtly linked to proliferation [6]; and, probes for lncRNAs *H19* and *HOTAIR* that we previously found to show a relationship with breast cancer by RNA ISH [11] were also selected for ISH use.

Given sample numbers, RNA ISH carcinoma staining was scored on a binary basis: Negative <10% cells stained; Positive 10% cells stained. DCIS staining was scored on a binary scale and also fourfold: 0–1, 2–9%, 10–50%, 51–100%. Additional descriptors noted included: staining location (tumor, stroma, nucleus, cytoplasm); signal pattern (weak, medium, strong, dots [single, dual, multiple], clusters [cells or nuclei densely stained]).

Microscopy Imaging

Specimens were imaged using an Aperio VERSA whole slide scanner (Leica Biosystems, Inc., Buffalo Grove, IL). H&E slides were scanned with a 20x and all other slides with a 40x objective lens. The .SCN image files were opened using Aperio ImageScope software and

focal TMA regions were selected for incorporation into the figure images. No post-scanning image alterations were made.

RNA ISH Statistical Analyses

Statistical analyses of RNA ISH data in relation to clinical data included SAS® 9.3 software (SAS Institute) and GraphPad InStat Software (La Jolla, CA) to compare the percent of samples from patients with positive (≥10%) staining in the low vs. intermediate and high RS groups. Cox regression was used to assess the association between the degree of staining (fourfold scale) and the risk of recurrence in DCIS patients.

RESULTS

Microarray Data Analyses

The comparison of low vs. intermediate/high RS values (TNBC samples excluded from comparison) was performed by Arraystar, Inc. Six lncRNAs were significantly upregulated in low RS compared to intermediate/high and one lncRNA was down-regulated in low RS relative to intermediate/high (Table 3A). Four mRNAs were significantly upregulated and six were significantly down-regulated in low RS vs. intermediate/high RS specimens (Table 3B).

Tissue Microarray RNA *In Situ* Hybridization

Invasive Breast Cancer—The lncRNA probes *BBC3-1*, *FER3*, *RAD21-AS1*, and *ZEB1-2* stained completely negative (<1%) among all samples (and consequently were not tested on DCIS specimens). Other staining data are summarized in Table 4 that shows positive (≥10%) staining data for the different marker sets relative to RS values. Qualitative differences in mRNA staining were noted among low, intermediate and high RS invasive tumors (Figure 1 and S1). As scored <10% negative vs. ≥10% positive, a significant association of the *HOTAIR* marker with intermediate/high RS tumors was noted ($P=0.0152$ [8/10 vs. 1/8; Fisher's Exact Test])

Two triple negative breast cancers, including one metaplastic carcinoma were also included among the samples. Of note, the proliferation panel and *MKI67* stained considerably more strongly in non-metaplastic TNBC than in the metaplastic breast cancer. The non-proliferation (Mammostrat) probe panel and especially lncRNA *H19* stained more strongly in metaplastic TNBC. *GLO1*, *GLTSCR2* and *TGFB1* stained slightly more strongly in the non-metaplastic TNBC; *TLR-2* and *HOTAIR* stained similarly. Selected TNBC staining data are shown in Figure 2.

Ductal Carcinoma In Situ—DCIS data are summarized in Tables 5 and 6 (complete data are provided in Supporting Information Tables S1 and S2). Figure 3 highlights selected staining patterns; (five case studies are shown in Figure S2). Approximately half of the DCIS lesions stained positive with the proliferation marker cocktail and nearly a third with the 'non-proliferation' panel cocktail. There were positive staining associations for the 'non-proliferation' panel cocktail and *TGFB1* and time to a subsequent event after DCIS (Table 6). Additionally, the proliferation panel pooled probe (*BIRC5*, *Cyclin B1*, *MKI67*, *MYBL2*,

STK15) 10% staining showed a significantly increasing trend across DCIS grade: Low, 0/4 (0%); Intermediate, 9/48 (18.75%); High, 11/29 (37.93%) [$P=0.0269$, Mantel-Haenszel Chi-Square test]. No other significant associations were found in relation to DCIS grade or DCIS size (Tables S1/2).

DISCUSSION

This study investigated a novel approach to recurrence risk marker discovery by combining microarray interrogation of breast carcinomas pre-stratified for risk by 21-gene assay and pathology review with lncRNA and mRNA ISH of breast carcinoma and DCIS TMAs and also included novel ISH of proliferation and non-proliferation probe panels. Seventeen markers were found to be differentially expressed: 7 lncRNAs, *AC078802.1-2*, *BBC3-1*, *CHURC1-FNTB-1*, *FER3*, *RAD21-AS1-1*, *SLC39A8-1*, and *ZEB1-2* (Table 3A), none of which have current citations in PubMed; and, 10 mRNAs, upregulated: *GLO1*, *GLTSCR2*, *TGFB1*, *RAB34*; downregulated: *IL2RA*, *INTS6*, *MXD1*, *RAB5B*, *TCF20 (SPBP)*, *TLR2* (Table 3B); associations with breast or other cancers have previously been reported for all 10 [12–21]. Of the upregulated mRNAs, *TGFB1* and *RAB34* are commonly cited as associated with proliferation and of the downregulated group *INTS6* is cited as a tumor suppressor gene (search and review of the NCBI gene database <https://www.ncbi.nlm.nih.gov/gene>). That seven of the 10 genes implicated in the recurrence phenotype are not overtly effectors of proliferation or suppression supports investigation of risk profiling algorithms involving diverse markers.

QRT-PCR validation of the microarray implicated markers was beyond the resources available for this study. Instead, validation was attempted for eight of the microarray implicated markers by RNA ISH. Absence of staining for the four lncRNA targets could simply be due to low level expression sub-threshold to the detection limits of the RNAscope ISH method. Ideally, RNAscope ISH utilizes 20 pairs of oligoprobes (“ZZs”) [9]. In the case of probes for *lnc-BBC-3* and *lnc-ZEB1-2* the shortness of the target sequences (Table 3A) limited the probe design to 9 ZZs and 8 ZZs respectively, which may have compromised assay analytical sensitivity.

Previously, we reported a continuum of increasing ISH expression of lncRNAs *H19* and *HOTAIR* from normal breast to DCIS to invasive disease [11]. The data in this study found no significant difference in *H19* expression among the carcinomas; however, *HOTAIR* expression showed an association with higher RS (Table 4) consistent with *in silico/in vitro* data [22]. *H19* RNA staining, as in our prior study, is notable for its streaking appearance in stromal tissues although ‘dot’ signals are also appreciable in individual tumor cells (Figures 1, 2 and S1). The highly abundant *H19* staining in the metaplastic TNBC specimen (Figure 2) is the strongest noted among the 52 breast cancers previously assayed [11] as well as the 20 current samples. Whereas in other *H19* positive breast tumors staining appears at tumor-stromal interfaces, in the metaplastic TNBC *H19* haloed cells with an intense ring of staining possibly located to the tumor cell membrane; further investigation of *H19* significance in the etiology of and potential as a marker for these rare tumors is warranted.

RNA ISH staining for the four mRNAs (*GLO1*, *GLTSCR2*, *TGFB1*, *TLR2*) selected did not show correspondence with the carcinoma microarray data (Table 4). This finding may be indicative of ISH having insufficient resolution to measure the fold changes (Table 3B), and/or differentials between assay endpoints. Microarray assays are quantitative tests whereas visual qualitative RNA ISH scoring may be impacted by observer bias and inability to quantify more subtle staining differences. The carcinoma and DCIS case studies shown in Supplementary Figures 1 and 2 are suggestive of qualitative staining differences. Binary or four-fold scoring may be inadequate to capture significant biologic differences. Microscopy imaging and dedicated software packages analyses may enable quantitative adequacy. Among the DCIS cohort (Tables 5 and 6) *TGFB1* RNA ISH staining shows potential utility: *TGFB1* expression was significantly associated with years to any recurrence event, an ipsilateral event, or invasion specifically (Table 6), consistent with literature showing that TGF- β 1 may promote an invasion-permissive microenvironment [23].

The proliferation marker set *BIRC5*, *Cyclin B1*, *MKI67*, *MYBL2*, *STK15* is a critical component of Oncotype DX panels for cancer or DCIS RS estimation [4, 10]. It was impractical in this study to assay each marker individually. The group was tested as a cocktail for tentative exploration of the hypothesis that a breast carcinoma or DCIS that stained negative or weakly would have a low RS or favorable outcomes and therefore might be excluded from referral for Oncotype DX testing. Relative to the RNA ISH stain grading employed in this study, the hypothesis is not supported (Tables 4–6, Figures 1, 3, S1, S2). Assay of a larger cancer cohort is warranted given that proliferation panel staining showed an association with increasing DCIS grade ($P=0.0269$). In contrast *MKI67* mRNA ISH alone did not show a direct relationship to DCIS grade possibly indicative of limiting sensitivity of *MKI67* ISH (or Ki-67 IHC) for proliferative index. Also of note, the metaplastic TNBC stained weakly with *MKI67* and with the proliferation panel; staining for these was much stronger in the other TNBC (and other cancers in the TMA) (Figure 2). As metaplastic breast cancer is known as an aggressive condition with a high risk for recurrence [24], these data are again suggestive of a distinct etiology for metaplastic TNBC and a requirement for diverse markers to define tumor aggressiveness.

The Mammostrat assay (IHC for CEACAM5, HTF9C, NDRG1, p53, SLC7A5) was developed specifically to screen for markers that are independent of one another and that do not directly measure either proliferation or hormone receptor status [6]. The IHC test was available via a proprietary referral laboratory performed and interpreted by Clariant Diagnostic Services, Inc., (Aliso Viejo, CA); further to a takeover by NeoGenomics, Inc. (Fort Myers, FL) the test is no longer available. A number of publications reported utility for this assay [6, 25, 26]. RNA ISH staining patterns for the gene analogs of the panel did not show a significant relationship to cancer RS values; one of the highest levels of staining was noted in a low RS tumor and there was only weak staining in intermediate and higher RS tumors suggesting limited utility for RNA ISH for this panel in pre-screening cancer specimens for Oncotype DX testing. However, among the DCIS cohort, non-proliferation panel RNA staining was significantly associated with recurrence events whereas the proliferation cocktail was not (Table 6). Staining was strong in both TNBC specimens, the more so in the metaplastic sample and somewhat the inverse of the proliferation panel staining pattern.

Comparing staining patterns, *MKI67* and the proliferation panel tended to show a checkered staining distribution among carcinomas, and in DCIS tended to align to the basal cell compartment. In contrast, the non-proliferation panel showed a more diffuse staining pattern in carcinomas, and in DCIS stained all compartments, tending to stain more strongly in the luminal region (Figures 1–3, Supplementary Figures 1 and 2). Interestingly, staining positive for both the proliferation and non-proliferation panels was not a marker for DCIS recurrence risk whereas staining for one or the other was associated with recurrence risk (Table 6, Tables S1/S2) suggestive of independent pathways to aggressiveness and likely reflective of the heterogeneous nature of cancer; eleven breast cancer molecular subtypes have been described [27]; four molecular subtypes have been reported for TNBC [28]. The IHC4 test estimates recurrence by inter-relating ER, PR, Her2 and Ki-67 as: $94.7 * (0.100 * ER_{10} + 0.079 * PgR_{10} + 0.586 * HER2/neu_{0/1} + 0.240 * Ln[1 + 10 * Ki-67_{\%pos}])$ [7]. The Mammostrat IHC test also devised an empirical calculation for recurrence: $(1.542 \times SLC7A5) + (1.124 \times p53) + (1.058 \times NDRG1) + (0.712 \times HTF9C) + (0.504 \times CEACAM5) - 0.864$ [6, 24, 25]. The addition of clinical data into IHC4 risk assessment improves recurrence estimation [29, 30, 31, 32]. Molecular tests such as Oncotype DX, Breast Cancer Index, EndoPredict, Prosigna and Mammoprint compound multiple biomarkers according to empirically discovered equations to assign risk (1–5) and are in common clinical use. IHC or RNA ISH remain as attractive diagnostic possibilities because of the ability to relate staining data to histopathological and other clinical features. The challenge for developing new IHC or RNA ISH predictive tests lies in identifying and inter-relating panels of diverse markers (including tumor immune and microenvironment targets [33]) by way of innovative imaging techniques, objective staining measurements and thresholds relative to pathology, molecular subtypes and clinical outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure 1. H&E and RNA ISH data (MKI67, proliferation panel and non-proliferation panel) from three breast carcinoma specimens highlighting differential staining. (Patients 3, 13, 16 see Table 1).

Figure 2. TNBC staining. Markers showing different RNA ISH staining patterns between two triple negative breast carcinomas, one metaplastic (Patients 19 & 20 see Table 1). Scale bar: 50 μ m.

Figure 3.

DCIS staining. Representative RNA ISH staining of three DCIS lesions (Cases A, B and C) for eight markers including positive control probe POLR2A. Arrows indicate low frequency signals. Scale bars: Case A, 200 μ m; Cases B and C 50 μ m except Case C: MKI67, 80 μ m. Case studies of five DCIS (low, intermediate and high grade) stained for eight markers are shown in Figure S2.

Table 1.

Breast carcinoma clinical data summary

Patient	Age	Diff.	Stage	Size (mm)	ER	PR	RS	10-YR R (%)	5-YR Follow-Up
1	64	W	T1b N0	8	>90	>90	0	3	Non-recur
2	57	P	T1c N0	17	>90	>90	0	3	Non-recur
3	59	W	T1c N0	15	>90	>90	1	4	Non-recur
4	77	P	T1c N0	17	98	98	8	6	Non-recur
5	66	M	T2 N1a	25	>90	>90	9	7	Non-recur
6	67	W	T1c N0	11	>90	90	10	7	Non-recur
7	64	M	T3 N0	54	>90	0	10	7	Non-recur
8	68	M	T1c N1 mi	15	>90	80	11	7	Non-recur
9	62	P	T1c N0	20	>90	40	27	17	Non-recur
10	59	P	T1c N0	40	>90	70	27	17	[†] Non-recur
11	71	P	T1c N0	15	90	20	30	20	Non-recur
12	75	P	T1c N0	12	90	0	32	22	Non-recur
13	73	P	T2 N1a	26	>90	2	34	23	[§] Non-recur
14	59	P	T2 Nx	19	>90	0	37	26	Non-recur
15	68	P	T2 N0	39	>1	30	59	34	Non-recur
16	63	P	T1c N0	15	90	90	Hi [†]	NA	Non-recur
17	64	P	ypT3 ypN0	70	80	0	Hi [†]	NA	Non-recur
18	75	P	ypT4b ypN3a	60	90	2	Hi [†]	NA	^R Recur
19	53	P	cT2 Nc0	40	0	2	TNBC	NA	[¶] Non-recur
20	65	P	cT3 Nc0	108	0	<1	TNBC(M)	NA	[•] Non-recur

Diff.; Differentiation, W, well; M, moderately; P, poorly; RS, Oncotype DX Recurrence Score: low 17, intermediate 18–30, high 31–100; 10-YR R (%), 10 year risk of recurrence percent; TNBC triple negative breast cancer; TNBC(M) – TNBC/metaplastic breast cancer;

[†] not tested by Oncotype DX; assessed and treated as high-risk for recurrence on the basis of clinical data.

[‡] Patient had prior cancer in same breast;

[§] subsequently uterine serous carcinoma;

[¶] ypT2 (25 mm) ypN0;

[•] ypT3 (55 mm) ypN0 after chemo after chemo;

^R Recur, cervical lymph nodes

Table 2.

Ductal carcinoma in situ clinical data summary

	N	%
Age at diagnosis		
<40	2	2.4
40–49	24	28.6
50–59	25	29.8
60–69	14	16.7
70–79	15	17.9
80+	2	2.4
Missing	2	2.4
Year at Diagnosis		
1994–1998	42	50.0
199–2003	28	33.3
2004–2008	11	13.1
2009–2012	1	1.2
Missing	2	2.4
Mode of detection		
Screen-detected	45	53.6
Symptom-detected	17	20.2
Missing	22	26.2
Grade		
Low	4	4.8
Intermediate	48	57.1
High	30	35.7
Missing	2	2.4
Size		
1 cm	44	52.4
1.1–2 cm	31	36.9
>2 cm	7	8.3
Missing	2	2.4
ER status		
Negative	7	8.3
Positive	10	11.9
Missing	67	79.8

Tables 3A.

LncRNAs differentially expressed in low vs. intermediate/high RS tumors

Low RS vs. int/high RS	P-value	Fold Change	RNA Length	LNCipedia name
Upregulated	0.0126	1.44	1908	<i>Lnc-FER-3</i>
Upregulated	0.0133	1.32	449	<i>Lnc-BBC3-1</i>
Upregulated	0.0016	1.25	479	<i>Lnc-SLC39A8-1:1</i>
Upregulated	0.0157	1.24	2010	<i>Lnc-RAD21-AS1-1:1</i>
Upregulated	0.0177	1.21	574	<i>Lnc-CHURC1-FNTB-1:4</i>
Upregulated	0.0479	1.21	541	<i>Lnc-AC078802.1-2:1</i>
Downregulated	0.031	1.30	427	<i>Lnc-ZEB1-2</i>

Table 3B.

mRNAs differentially expressed in low vs. intermediate/high RS tumors

Low RS vs. int/high RS	P-value	Fold Change	Gene Name
Upregulated	0.0480	1.29	<i>GLTSCR2 (NOP53)</i>
Upregulated	0.0289	1.24	<i>GLO1</i>
Upregulated	0.0359	1.23	<i>TGFB1</i>
Upregulated	0.0390	1.21	<i>RAB34</i>
Downregulated	0.0343	1.66	<i>INTS6</i>
Downregulated	0.0388	1.46	<i>TLR2</i>
Downregulated	0.0475	1.34	<i>MXD1</i>
Downregulated	0.0418	1.25	<i>RAB5B</i>
Downregulated	0.0317	1.24	<i>IL2RA</i>
Downregulated	0.0307	1.22	<i>TCF20</i>

Table 4.

RNA ISH data[†] for low, intermediate and high recurrence score invasive tumors (n=18)

Probe	Cells stained	Low RS (n=8)	Intermed. RS (n=3)	High RS (n=7)
Proliferation panel[‡]	Tumor	6 (75%)	3 (100%)	7 (100%)
<i>MKI67</i> mRNA	Tumor	2 (25.0%)	3 (100%)	5 (71.4%)
Non-proliferation panel[§]	Tumor	8 (100%)	3 (100%)	7 (100%)
<i>GLO 1</i> mRNA	Tumor	8 (100%)	3 (100%)	7 (100%)
<i>GLTSCR2</i> mRNA	Tumor	8 (100%)	3 (100%)	7 (100%)
<i>TGFB-1</i> mRNA	Tumor or Stroma	5 (62.5%)	0 (0%)	3 (42.9%)
<i>TLR-2</i> mRNA	Tumor or Stroma	2 (25.0%)	0 (0%)	3 (42.9%)
<i>H19</i> lncRNA	Stroma	7 (87.5%)	1 (33.3%)	7 (100%)
<i>HOTAIR</i> lncRNA	Tumor	1 (12.5%)	2 (66.6%)	6 (85.7%)

[†] Positive: 10% staining;

[‡] (*BIRC5*, *Cyclin B1*, *MKI67*, *MYBL2*, *STK15*);

[§] (*CEACAM5*, *HTF9C*, *NDRG1*, *SLC7A5*, *TP53*)

Table 5.mRNA ISH staining among DCIS Patients (N=74 – 84)[†]

Probe	Positive [‡]	Negative
Proliferation [§] Gp.	20 (24.1%)	63 (75.9%)
Non-Proliferation [¶] Gp.	20 (24.5%)	62 (75.6%)
Prolif. ¹ or Non-Prolif. [¶] Gp.	29 (35.4%)	53 (64.6%)
Prolif. & Non-Prolif. ^{§, ¶} Gps.	11 (14.9%)	63 (85.1%)
<i>MKI67</i>	5 (6.1%)	77 (93.9%)
<i>TGFB1</i>	5 (20.0%)	75 (80.0%)
<i>TLR2</i>	0	83 (100%)
<i>GLTSCR2</i>	47 (60.3%)	31 (39.7%)
<i>GLO1</i>	33 (42.9%)	44 (57.1%)

[†] N values vary due to TMA tissue spot drop-out 'missing data points';[‡] 10% staining.[§] (*BIRC5*, *Cyclin B1*, *MKI67*, *MYBL2*, *STK15*);[¶] (*CEACAM5*, *HTF9C*, *NDRG1*, *SLC7A5*, *TP53*)

Table 6.

DCIS mRNA staining: Significant risk associations with subsequent tumor events

	HR [†] (95% CI)	P-value [‡]
Years to any event: DCIS or invasive		
<i>TGFB1</i>	8.26 (3.22–21.16)	0.0001
Years to first invasive event		
<i>TGFB1</i>	7.85 (1.92–32.11)	<0.0001
Years to first ipsilateral event		
Non-Proliferation [§] Gp.	3.06 (1.48 – 6.35)	0.003
<i>TGFB1</i>	7.85 (1.92–32.11)	0.004
Years to first DCIS event		
Non-Proliferation [§] Gp.	2.13 (1.01 – 4.47)	0.047
Prolif.[¶] or Non-Prolif.[§] Gp. with > 10% staining: associations with outcome		
Any recurrence	1.98 (1.08–3.64)	0.027
DCIS	2.30 (1.13–4.50)	0.022
Ipsilateral	3.19 (1.62–1.43)	0.001

[†]Hazard ratio (HR) per unit increase in ordinal staining scale.[‡]P-value test for trend.[§](*BIRC5*, *Cyclin B1*, *MKI67*, *MYBL2*, *STK15*);[¶](*CEACAM5*, *HTF9C*, *NDRG1*, *SLC7A5*, *TP53*).

Note: DCIS patients with recurrences n=55. Complete data analyses tables are provide in Supporting Information Tables 1 and 2.