Detection of disseminated tumor cells in the bone marrow of breast cancer patients using multiplex gene expression measurements identifies new therapeutic targets in patients at high risk for the development of metastatic disease

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Abstract

Purpose—Disseminated tumor cells (DTCs) detected in the bone marrow of breast cancer patients identifies women at high risk of recurrence. DTCs are traditionally detected by immunocytochemical staining for cytokeratins or single gene expression measurements, which limit both specificity and sensitivity. We evaluated the Nanostring nCounter™ (NC) platform for multi-marker, gene expression-based detection and classification of DTCs in the bone marrow of breast cancer patients.

Experimental Design—Candidate genes exhibiting tumor cell specific expression were identified from microarray data sets and validated by qRT-PCR analysis in non-malignant human BM and identical samples spiked with predefined numbers of molecularly diverse breast tumor cell lines. Thirty-eight validated transcripts were designed for the nCounter™ platform and a subset of these transcripts was technically validated against qRT-PCR measurements using identical spiked bone marrow controls. Bilateral iliac crest bone marrow aspirates were collected and analyzed from twenty breast cancer patients, prior to neoadjuvant therapy, using the full 38 gene nCounter™ code set.

Results—Tumor cell specific gene expression by nCounter™ was detected with a sensitivity of one cancer cell per 1×10^6 nucleated bone marrow cells after optimization. Measurements were quantitative, log linear over a twenty-fold range, and correlated with qRT-PCR measurements.

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Using the nCounter™ 38-gene panel, 6 of 8 patients (75%) who developed metastatic disease had detectable expression of at least one transcript. Notably, three of these patients had detectable expression of *ERBB2* in their bone marrow, despite the fact that their corresponding primary tumors were HER2/ERBB2 negative and therefore did not receive trastuzumab therapy. Four of these patients also expressed the *PTCH1* receptor, a newly recognized therapeutic target based on hedgehog signaling pathway inhibition.

Conclusions—The presumptive detection and classification of DTCs in the bone marrow of breast cancer patients, based on sensitive and quantitative, multi-marker detection of gene expression using the nCounter™ platform provides an opportunity to both predict early distant recurrence and more importantly, identify opportunities for preventing the spread of disease based on the expression of unique, therapeutically actionable gene targets.

Translational relevance—This study demonstrates the application of a new technology for multiplexed gene expression-based detection of disseminated tumor cells in the bone marrow of breast cancer patients, and identifies at least two therapeutically targetable genes that are frequently expressed in BM of patients who develop metastatic disease.

Keywords

breast cancer; disseminated tumor cells; gene expression; Nanostring nCounter

Introduction

Approximately 40,000 women die from breast cancer each year as a consequence of metastatic disease development. Data suggests that only a small, unique subset of cells within a primary breast tumor (less than 1%) possess metastatic potential [1,2]. Furthermore, molecular profiles of primary, heterogeneous tumors may demonstrate limited resemblance to those tumor cells that eventually progress to metastatic foci [3]. Clinically, therapies that reduce primary tumor mass by simply eliminating proliferating cells often fail to cure patients, particularly with respect to metastatic disease [4]. To develop new therapeutic strategies to control and monitor distant disease development, it is essential to identify and target the intermediary cells in the metastatic process since these cells likely have biological behavior and therapeutic vulnerabilities which differ from the primary tumor [5].

Disseminated tumor cells (DTCs) can be detected in the bone marrow (BM) of 30-40% of early stage breast cancer patients[6]. Several large multi-institutional clinical trials have documented that the presence of BM DTCs at the time of diagnosis identifies women at high risk of recurrence. Those women with detectable DTC after completion of all adjuvant therapy are at higher risk of recurrent disease development than those women with no detectable DTC in their BM[7,8]. The proposed biological basis for this clinical observation is that BM serves as a reservoir that allows DTCs to adapt and disseminate to other organs[9]. Alternatively, it is possible that clinically detected BM DTCs may be a marker of body-wide dissemination of tumor cells rather than the body's only long-term reservoir[10-12]. Regardless, DTC detection allows the identification of women at high risk of recurrence and the ability to monitor response in those women with clinically undetectable disease.

Although the detection of DTCs in breast cancer is still investigational, its incorporation into clinical management of breast cancer patients is currently the focus of much research. Standardized guidelines have been published for the immnocytological (ICC) detection of DTCs[13], which is the current gold standard and has a reported sensitivity ranging from 1 DTC in $10^5 - 10^6$ leukocytes[14]. Antibodies against epithelial-specific antigens such as cytokeratins (CK) or EpCAM, surface adhesion molecules, or growth factor receptors have been used for the detection of carcinoma cells due to the absence of more tumor-specific target antigens[15]. Other studies have assessed DTC detection using assays that detect single epithelial- or organ-specific transcripts such as cytokeratin 19 (*KRT19*), *MUC1*[16], urokinase-type plasminogen activator receptor (*uPAR*) [17], EpCAM (*TACSTD1*), and mammaglobin (*SCGB2A2*)[18-23]. Expression of *SCGB2A2*, *KRT19*, and *TWIST1* in the BM of breast cancer patients have individually demonstrated prognostic significance[24-27]. However, because individual gene expression is not always absolutely tumor-specific, false positive results can be observed due to low level transcription of epithelial or breast tissue-specific genes in normal BM cells [28,29]. Conversely, given the known molecular heterogeneity of DTCs, it is unlikely that a single gene marker will be suitable for the detection of DTCs in all breast cancer patients. For example, *KRT19* is not expressed by all DTCs; its expression is often lost in breast cancer cells during tumor progression and epithelial to mesenchymal transition (EMT) [30,31].

A multi-marker assay approach has the potential to overcome some of the above concerns by allowing the simultaneous, composite detection of multiple genes in a single sample [32-34,26]. Analysis of DTC-associated molecular profiles could improve both sensitivity and specificity of DTC detection itself, and also provide prognostic or therapeutically predictive information for patients as well. The Nanostring nCounter™ (NC) assay platform allows for the simultaneous, quantitative detection of multiple (up to 600) nucleic acid targets in a single sample with a sensitivity and reproducibility comparable to qRT-PCR [35,36]. Accordingly, we have mined existing gene expression microarray data to identify and validate gene transcripts whose expression is specifically associated with breast tumor cells and absent in normal bone marrow cellular constituents. We have adopted a panel of 38 of these genes to the NC platform, and in this study, demonstrate how the assay can be used with sufficient sensitivity to profile clinical BM samples from breast cancer patients to detect and potentially classify rare BM-associated DTCs. In a cohort of twenty patients, eight of whom had distant metastatic recurrence within 48 months, composite patterns of gene expression identified patients more likely to develop metastatic disease and who might be candidates for targeted therapeutics directed to both *ERBB2* and *PTCH1* gene expression.

Methods

Study Population

Control bone marrow specimens were collected from healthy female volunteers after informed consent. DTC-positive bone marrow previously assessed by immunohistochemistry was obtained retrospectively from a biospecimen collection associated with a previously reported therapeutic trial[37]. Specimens for the test clinical cohort were also obtained retrospectively from an independent, ongoing IRB approved bone

marrow and tissue banking breast cancer protocol at our institution. All patient bone marrow aspirates were collected prior to the initiation of any therapy. An overview of clinical samples used in this study is shown in Supplemental Table 1.

Bone Marrow Aspiration, Processing, and RNA Isolation

For all breast cancer cases, twenty ml of bone marrow was collected from the right and left anterior iliac crests, subjected to hypotonic RBC lysis, washing, and nucleated cell counting within two hours from the time of collection. For controls, bone marrow was harvested unilaterally. For whole bone marrow analysis, 5×10^6 nucleated cells were pelleted and immediately snap frozen for subsequent RNA isolation. For mononuclear cell enrichment, whole bone marrow was diluted five-fold in RPMI media and processed using a standard Ficoll-Hypaque gradient technique. The enriched mononuclear cell fraction was then counted and 5×10^6 mononuclear cells were pelleted and snap frozen for subsequent RNA isolation. SKBR3 (ER-/Her2+), MDA/MB231 (ER-/Her2-), and ZR75 (ER+/Her2-) human breast cancer cells were trypsinized from plates during log growth phase, washed, counted, diluted into the prepared nucleated cell fraction of normal human BM samples at incremental five-fold dilutions from 1:1000 to 1:500,000 tumor cells per nucleated cell count, pelleted, and snap frozen for subsequent RNA isolation. All RNA isolations were performed from snap frozen cell pellets using Trizol reagent (Invitrogen) as previously described[27]. RNA was quantified by A_{260} using a Nanodrop spectrophotometer and qualitatively assessed using an Agilent bioanalyzer. All RNAs used for qRT-PCR or NC analysis demonstrated a RIN score of > 7.0.

qRT-PCR Analysis

SyBr green or TaqMan Assay-on-Demand primer/probe sets for indicated transcripts were purchased from Applied Biosystems. Primer/probe sequences are shown in Supplemental Table 2. One microgram of total RNA was reverse-transcribed and 1/100th of the cDNA template was used in each replicate qPCR reactions. Each reaction consisted of 1ul of cDNA, TaqMan Master Mix (Applied Biosystems), and 5× primer/probe set in a total volume of 20ul, following the manufacturer's standard protocol. For each transcript/sample, duplicate or triplicate reactions were performed in an ABI 7500 FAST Sequence Detection System. Replicated sample assays with a cycle threshold (CT) value difference of greater than 1.5 (outliers) were excluded from analysis. Each run with a gene primer set was normalized to CT values of GAPDH primer set on the same cDNA sample. This value was in turn normalized across the entire sample set by using the average delta CT from an identical analysis using a set of four normal volunteer BM specimens, using the ddCT method [38]. In samples where a specific transcript was not detected (CT>40), the fold difference in expression was defined as 1.0 (equal to that of the normal bone BM average).

Nanostring nCounter Assay

The nCounter assay was performed with the 38 gene codeset as previously described[8] with the exception that input RNA was increased from 0.5 ug to 5 ug to increase the sensitivity for detecting transcripts expressed only by rare DTC populations. Eleven positive and nine negative controls spikes were used to calibrate the assay. Mean hybridization counts of the negative controls were subtracted from all other transcript counts to correct for

background[34]. Patient samples were run in duplicate or triplicate and the average value taken for analysis. Reference values for each gene were calculated from cohorts of 8 and 11 bone marrow samples from healthy female volunteers. All expression values were normalized based on the average expression of three internal control transcripts (*TBP, RPR0, POL2RA*). For binary analysis, a sample was considered positive for gene expression if hybridization counts were 2 standard deviations above the mean of the healthy control bone marrow population.

Statistical Methods

Spearman's rank correlation coefficient was calculated on PCR and NC data after removing values at the extreme of measurement. Coefficient of variation was calculated on normal bone marrow specimens to assess the variance within controls. Assessment of variance within patient samples was carried out treating differences between patient samples as signal and differences within patient samples as noise.

Results

Target Gene Selection

To identify gene transcripts that could potentially serve as specific markers for DTCs in the context of patient bone marrow, we analyzed gene expression microarray data from 80 primary breast tumors, 79 serial bone marrow samples with and without known DTC involvement (based on immunocytochemistry) from 25 breast cancer patients, 5 bone marrow samples from healthy volunteers, as well as additional breast tumor data sets in literature[39,27,40]. Comparative expression analysis of these data sets identified genes that had detectable microarray-based expression in either primary tumors and/or ICC-positive BM specimens, but were not detectable in similar data sets from healthy volunteer bone marrow or in ICC-negative breast cancer patient bone marrow. A total of sixty-nine genes met these criteria (Supplemental Table 3). To further validate their tumor-specific expression, each gene was quantified by qRT-PCR using a set of healthy volunteer bone marrow samples and three exemplar breast tumor cell lines of differing molecular subtypes: SKBR3 (*HER2*+), ZR75 (ER+), or MDAMB231 (*HER2*-/ER-). Of the 69 genes chosen, 27 genes met the criteria of undetectable or nearly undetectable expression (defined as Ct > 37) in healthy volunteer BM and a minimum of twenty-fold (range 20-18,000) higher expression in at least one of three breast cancer cell lines relative to the healthy control population. Eleven genes (*CCND1, ESR1, FGFR4, Gli2, Gli3, GSC, IGFBP4, PDGFRB, PTCH1, SMO, SNAI1*) were expressed at less than twenty fold over that in healthy BM but were included in the panel due to their biological and/or clinical relevance (Supplemental Table 4). Figure 1 lists the genes that comprise the full 38-gene panel, along with their biological process associations.

nCounter Performance Comparison

Transcript expression analysis by qualitative or semi-quantitative RT-PCR remains the most documented approach for molecular detection of breast cancer DTCs, primarily because of the requisite sensitivity that amplification methods can provide. To determine whether the non-amplification based nCounter platform could have sufficient sensitivity to detect gene

expression from rare DTCs amidst a high background of normal bone marrow constituents, we examined the expression of a subset of nine genes (*Twist1*[27], *SNAI2*[41], *EPCAM*[42,43], *SCGB2A2*[44], *EGFR*[45], *PITX2*[46], *S100A3*[27], *KRT17*, and *KRT19*[47]) from the 38 gene panel in the three exemplar breast cancer cell lines serially diluted into normal human BM. As shown in Table 1 and Figure 2, expression measurements were quantitative and varied over a 20-fold linear dynamic range. Consistent with previous reports [48,49], not all of the nine genes were detected in each of the cell lines, demonstrating the necessity of a multi-gene marker panel for detecting DTC expression from cells of varying molecular phenotypes. Although *KRT19* transcript was detected in BM samples spiked with each of the cell lines, each cell line demonstrated a unique pattern of expression of one or more genes.

In parallel, the same sample dilution series were used for single gene qRT-PCR expression analysis. For those genes with detectable expression by nCounter assay, expression levels measured by qRT-PCR and nCounter demonstrated good correlation over the full dynamic range (Figure 2). Spearman's rank correlation coefficient between qRT-PCR (expressed as fold increase over expression in non-malignant bone marrow) and nCounter (expressed as hybridization counts over that from non-malignant bone marrow) for *KRT19*, *S100A3*, *SCGB2A2*, *TACSTD1*, and *TWIST1* ranged from .80 to .99 in those cell lines that expressed the individual genes.

Using 0.5 ug of input RNA, *KRT19* expression could be detected at a level of one cancer cell per 100,000 nucleated BM cells. However, the limits of reported detection using conventional immunocytochemistry have been reported at one cancer cell per 1 million nucleated bone marrow cells, theoretically ten-times more sensitive[50]. Therefore, to increase the sensitivity of the assay, we modified the standard protocol to perform both mononuclear cell enrichment (a standard procedure employed for ICC analysis) and increased input RNA to five micrograms, a quantity of analyte that is still readily obtainable from a routine bone marrow aspiration. Mononuclear cell enrichment increased expression signal levels by approximately two fold while, as expected, a ten fold increase in RNA input increased expression signals proportionally by ten fold as well (Supplemental Table 5). Not surprisingly, some transcripts that previously demonstrated nominal expression in normal bone marrow cell constituents (*SNAI2*, *TACSTD1*, *S100A3*, *KRT17*) had higher background expression using the modified protocol, while background expression of several other transcripts (*KRT19*, *EGFR*, *TWIST1*, *SCGB2A2*, *PITX2*) was unaltered by the protocol modification. Importantly, the protocol modification allowed us to achieve a theoretical sensitivity of one DTC per one million nucleated BM cells, the generally accepted limit of sensitivity observed with the 'gold-standard' ICC detection.

Analysis of Bone Marrow from Breast Cancer Patients

To demonstrate the clinical applicability of this method, the nCounter nine-gene panel was first tested in five bone marrow samples collected from patients with stage II/III breast cancer prior to any treatment, who had previously documented DTCs in their bone marrow by ICC (Table 2). A 'normal range' for bone marrow expression of each gene was established by analyzing bone marrow from eight female healthy volunteers. Intra-class

correlation coefficient which compares within patient variance to total noise variance was calculated for the nine genes and this ranged from .622 to .909. At least one gene transcript was detected above background bone marrow signal in four of the five breast cancer bone marrow samples. Although, from this small sample set, a composite pattern of expression based on primary tumor molecular phenotype was not obvious, two ER-/Her2- breast cancer cases had detectable bone marrow expression of several genes known to be expressed in this subtype of primary breast cancer (*EPCAM, SNAI2, KRT17*). Interestingly, *KRT19* gene expression, a molecular marker often used for detection of DTCs, was detected in only one of the five patient specimens.

Having documented performance characteristics of the assay and applicability to primary bone marrow samples from breast cancer patients with known DTCs, we analyzed retrospectively collected bilateral bone marrow from an independent cohort of twenty clinical stage II/III breast cancer patients and an additional reference set of 11 healthy female volunteers, using the entire 38 gene panel. Eight of twenty patients developed metastatic disease within 2-48 months of primary diagnosis (mean 23 months), while twelve had no evidence of metastatic disease at 3-5 years of follow-up. As shown in Figure 3, 20 transcripts of the 38 gene panel were expressed above background in at least one bone marrow sample, while at least one transcript was expressed above background in 17 patients (85%). None of the bone marrow specimens in this cohort had detectable expression of *KRT19* and only four patients (20%) had detectable expression of *EPCAM*, two genes commonly used for the molecular detection or selection of DTCs. Most patients demonstrated unique patterns of gene expression in bone marrow. However, with only a few notable exceptions (Figure 3, patient 3709) patterns of gene expression were highly concordant between right and left bone marrow samples from the same patient in 11 of 20 cases.

Unsupervised, hierarchical clustering of expression data demonstrated no obvious subclassification of cases, particularly with regard to molecular phenotype of the primary tumor or the occurrence of eventual metastases. Although 6 of the 8 patients (75%) who developed metastatic disease had detectable expression of at least one of the 38 genes in their bone marrow, patients who remained disease-free had a mean number of detectable transcripts that was not significantly different than that of patients with metastatic relapse. However, at the level of individual gene analysis, two transcripts (*SNAIL2 and LAMB1*) demonstrated a significantly different level of expression in bone marrow from patients who relapsed versus those that remained disease-free (p = .0013 and .0014 respectively with correction for multiple comparisons). One additional transcript from the 38-gene signature (*IGFBP4*) also demonstrated expression differences that were close to significance between disease free and metastatic patient populations (p=.058). Notably, several genes that are known targets for therapeutic intervention: *ERBB2*, *PTCH1*, and *PDGFRB*, were also expressed in the bone marrow of patients who eventually developed metastatic disease. Of the eight patients with metastatic relapse, one or more of these therapeutic targets were expressed in four patients (50%). Four of six patients with *PTCH1* positive marrow eventually developed metastatic disease and therefore, could have potentially benefited from adjuvant therapy directed to gene in the Hedgehog pathway. Three of four patients (Figure 4) with HER2 negative tumors and ERBB2 positive DTC eventually developed metastasis,

while none of the patients with HER2 positive tumors and ERBB2 positive bone marrow who received therapy with traztuzumab developed metastasis. For this set of patients, there was good concordance between detection of selected genes by PCR and NS (Supplemental Table 7)

Discussion

The presence of DTCs in the bone marrow of breast cancer patient has important prognostic and therapeutic implications. Adjuvant therapy administration is generally based on the primary tumor characteristics and lymph node status. However, those women with no detectable disease in their lymph nodes or bone marrow remain at very low risk of recurrence and may not benefit from adjuvant treatment[51]. Conversely, patients who have completed adjuvant therapy, but retain residual micro-metastatic disease could benefit from further therapy. DTCs are heterogeneous [52,53], can persist for years and remain a predictor for disease recurrence[54], may exhibit a stem cell-like chemotherapy resistant phenotype, [55] and likely have varying metastatic capabilities. Patients with detectable DTCs in their BM after chemotherapy treatment have a particularly poor prognosis indicating that chemotherapy does not eliminate all DTCs and that those subpopulations of DTCs which survive cytotoxic chemotherapy have a high metastatic potential[56]. Persistence of DTCs in BM years after diagnosis and initial therapy has been shown to be an indicator of subsequent systemic treatment failure [7,8,32]. Persistence or disappearance of DTCs after systemic treatment could therefore be used as a surrogate marker of treatment response [57]. Assays that detect and molecularly classify residual micro-metastatic disease could greatly benefit and guide the treatment of patients.

Currently the 'gold 'standard' for DTC detection is ICC which is laborious and often limited in sensitivity and specificity given the heterogeneity of DTCs. Molecular assays based on detection of single, 'epithelial specific' transcripts such as *EPCAM* or *KRT19* often perform no better than ICC based methods[58]. Given the known heterogeneity of DTCs, multiplexed biomarkers could provide improved sensitivity and specificity for detection and classification, but the ability to effectively multiplex multiple markers in a single reaction while maintaining high specificity and sensitivity for each marker in clinical specimens has been limited and few studies have been published regarding the genomic profiling of DTCs[14]. A major challenge lies in identifying a panel of genes that are biologically relevant, representative of all possible breast cancer molecular subtypes, and has very absent or minimal expression in non-malignant bone marrow constituents. Thus, a gene such as *CD44*, whose expression is related specifically to the breast cancer stem cell phenotype and metastases, is not useful in this assay since it is expressed in multiple hemapoietic cells[58]

The Nanostring nCounter platform provides an attractive approach for performing multiplexed gene expression assays in a single reaction and can quantitatively detect expression of up to 600 gene transcripts in a single reaction with similar sensitivity and reproducibility as amplification based methods[34]. Most importantly, the assay does not require high quality RNA and the direct readout of transcript copy number provides a higher precision for detecting gene expression originating from minor cell populations. In fact, we were surprised to find in our validation studies that the assay could detect minimally to

moderately abundant transcripts specifically emanating from as few as one tumor cell in a background of one million bone marrow cells, comparable to current ICC detection methods. This sensitivity was facilitated by enrichment of the mononuclear cell fraction of bone marrow, increasing assay RNA input by ten-fold without signal saturation, and careful selection of transcripts which demonstrated absent or minimal expression in bone marrow cell populations.

Despite selecting target transcripts which were expressed in one or more of three archetypical breast tumor cell lines of varying molecular phenotype, several of the genes in our 38-gene panel were not detected in the twenty cases of primary bone marrow from breast cancer patients analyzed in this study. It is possible that the expression of many of these genes is restricted to tumor cell lines or that our patient population in this study was too small to fully sample the diversity of DTC molecular phenotypes. For example we found that 1 of 5 samples in our first validation cohort was *TWIST1* expression positive, but none of the twenty samples in the second cohort had detectable levels of expression above background. In this regard, the flexibility of easily introducing or removing transcript probes in an iterative design approach for the nCounter assay is proved particularly useful for the current study and for future studies will allow the easy introduction of new targetable genes.

Conversely, we found that 65% of patients in our patient cohort demonstrated expression of at least one transcript in at least one of their bilateral bone marrow specimens. This far exceeds previously reported positivity based upon ICC detection of DTCs and clearly, many patients with a positive gene signature remained relapse free for more than five years. There are several possible explanations for this result. First, although gene expression levels were referenced to a population of healthy female controls, it is possible that the reference population size was not sufficiently large to fully capture inter-individual variability that may exist in healthy bone marrow. Second, since it is not possible to specifically equate expression from the 38-gene panel with the presence of tumor cells, gene expression in some patients could originate from rare bone marrow cell elements specifically present in the cancer patient population. Such a cancer-specific, 'stromal response' could still be biologically and clinically significant, even if it is not indicative of actual DTCs residing in the bone marrow. Finally, it is possible that 65% of our population do actually harbor DTCs in their bone marrow. Detection by cytokeratin ICC may underestimate the percentage of DTC positive patients, particularly for those DTCs that have undergone epithelial to mesenchymal transition and concomitant loss of cytokeratin expression [60]. Since only 6 of 17 patients (30%) with positive gene expression developed distant metastases within five years, it is possible that some bone marrow DTCs may have attenuated metastatic potential that can be directly related to their individual expression profiles.

In this regard, it was striking to see that patterns of DTC expression demonstrated such variability between patients. This cannot likely be attributed to biological noise since bilateral bone marrow aspirates demonstrated, except in a few cases, high concordance in expression patterns. In fact, it might be expected that in some patients, due to sampling bias, bilateral samplings may not be completely concordant. The molecular diversity of gene expression observed in this small subset of patients as well the biological processes that cancer cells undergo during their transition in the hematological system underscores the

need for a multiplexed gene expression panel. Furthermore, patterns of DTC gene expression did not appear to be directly related to the molecular phenotype of the primary tumor. In fact, several tumors that were Her2 negative corresponded to patient bone marrow samples with *ERBB2* expression, while two of the six patients with Her2 positive tumors had detectable expression of *ERBB2* in their bone marrow, and were also positive for other transcripts. Although the composite 38-gene expression pattern did not immediately classify patients based upon disease relapse and in fact, more than half of patients with a transcript positive bone marrow did not relapse, we identified several individual genes within the 38-gene signature that did reach a significant correlation with disease relapse- *LAMB1*, and *SNAI2*. In fact, *SNAI2* expression was inversely correlated with disease relapse suggesting that expression patterns may be related to 'favorable' as well as 'unfavorable' DTC molecular phenotypes.

Beyond providing prognostic information, several genes in the 38-gene panel are also relevant therapeutic targets- *ERBB2, EGFR, ESR1, PDGFB, PTCH1-* and were expressed in patient bone marrow specimens, often in non-overlapping patterns. This finding, together with the fact that expression of individual target genes such as *ERBB2* are often expressed discordantly in primary tumors and corresponding DTCs, suggests a utility for using DTC molecular profiles to 'personalize' adjuvant therapy in a prospective clinical trial setting. Although all specimens analyzed in the current study were collected only prior to therapy, this assay could also be applied to monitoring therapeutic efficacy in the adjuvant setting for those patients without radiographic or clinical evidence of disease. Bone marrow aspirations for DTCs performed before and following therapy and at times points during follow-up could lead to real-time assessments of therapeutic efficacy, and identify those patients in need of continued therapy. Subsets of DTCs that persist or are eliminated by therapy could be monitored and targeted. This type of assay platform for clinical samples could facilitate translation of multi-gene biomarkers into the clinic, identify women at high risk of breast cancer recurrence and provide guidance on tailored therapies based on the molecular profile of micro-metastatic breast tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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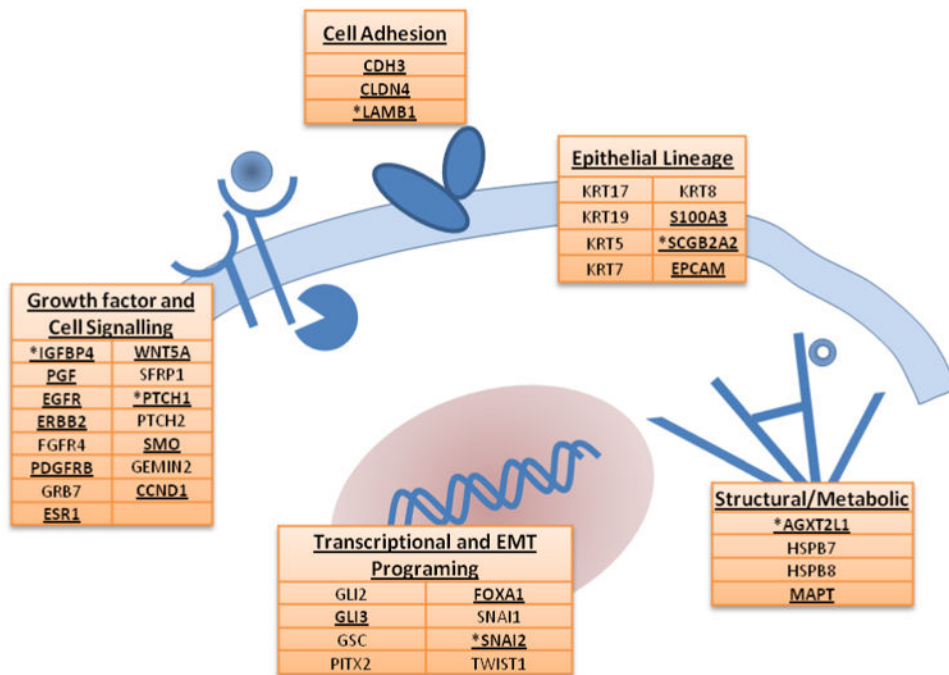
Cell Adhesion

CDH3
CLDN4
*LAMB1

Epithelial Lineage

KRT17	KRT8
KRT19	S100A3
KRT5	*SCGB2A2
KRT7	EPCAM

Growth factor and Cell Signalling

*IGFBP4	WNT5A
PGF	SFRP1
EGFR	*PTCH1
ERBB2	PTCH2
FGFR4	SMO
PDGFRB	GEMIN2
GRB7	CCND1
ESR1	

Transcriptional and EMT Programing

GLI2	FOXA1
GLI3	SNAI1
GSC	*SNAI2
PITX2	TWIST1

Structural/Metabolic

*AGXT2L1
HSPB7
HSPB8
MAPT

Figure 1.
Genes represented in the 38-gene DTC panel and their biological pathway associations. Transcripts detected in the twenty-patient pilot cohort are highlighted in **bold underline** and those associated with early metastatic recurrence are denoted by asterisks.
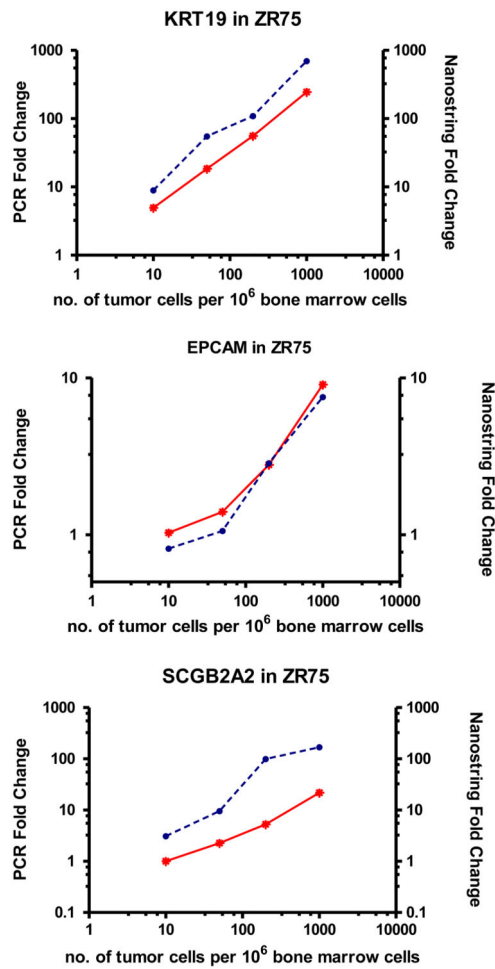
Figure 2.
nCounter and qRT-PCR detection of gene expression in control bone marrow spiked with increasing numbers of ZR75 breast tumor cells. Units on the right axis show the fold-difference of nCounter signal counts (red line) relative to normal bone marrow background. Units on left axis show fold-difference of expression (calculated by ddCT method) relative to normal bone marrow using qRT-PCR (blue dotted line). Axis are plotted in log scale.
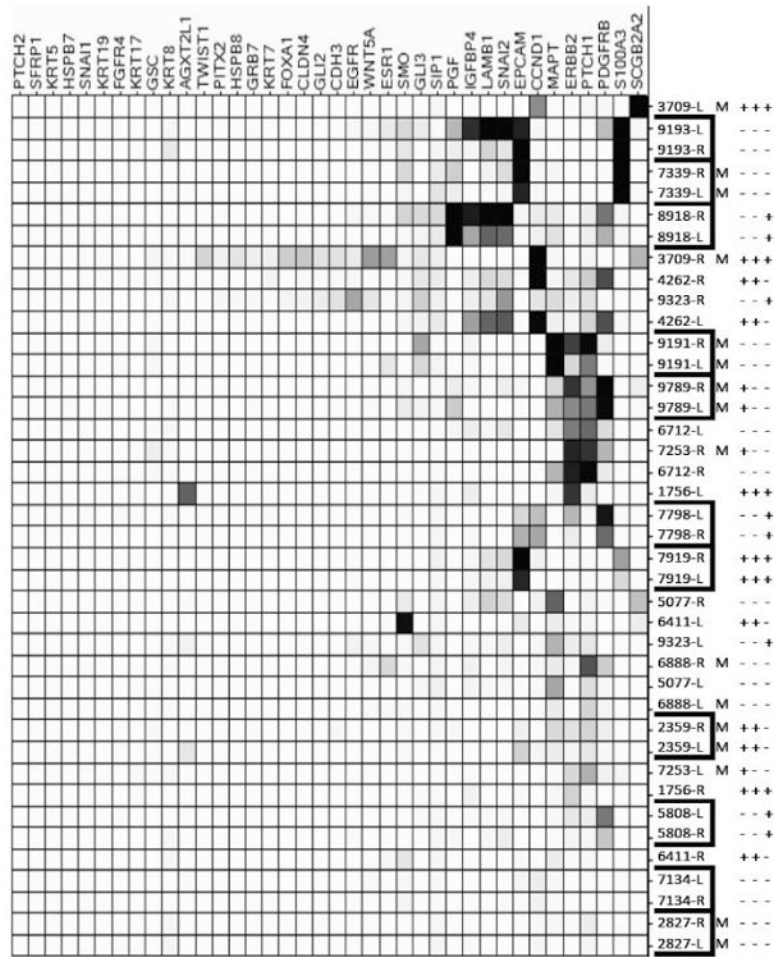
Figure 3. Unsupervised hierarchical clustering based on a 38-gene expression profile in bone marrow samples from breast cancer patients

Each row represents an individual sample (either left- L or right-R sampling) from 20 individual patients with a known breast tumor molecular phenotype (ER, PR, Her2 indicated with +/-). Eight patients developed metastatic disease (denoted by M) within 5 years of diagnosis. Each column represents relative expression of each gene in each sample. Expression is scaled based on number of SD above the mean of a set of 11 health control bone marrow samples analyzed in the same assay. Gene transcripts and samples are ordered and grouped based upon similar patterns of expression. Corresponding values for normalized hybridization counts for differentially expressed genes are shown in Supplemental Table 7.
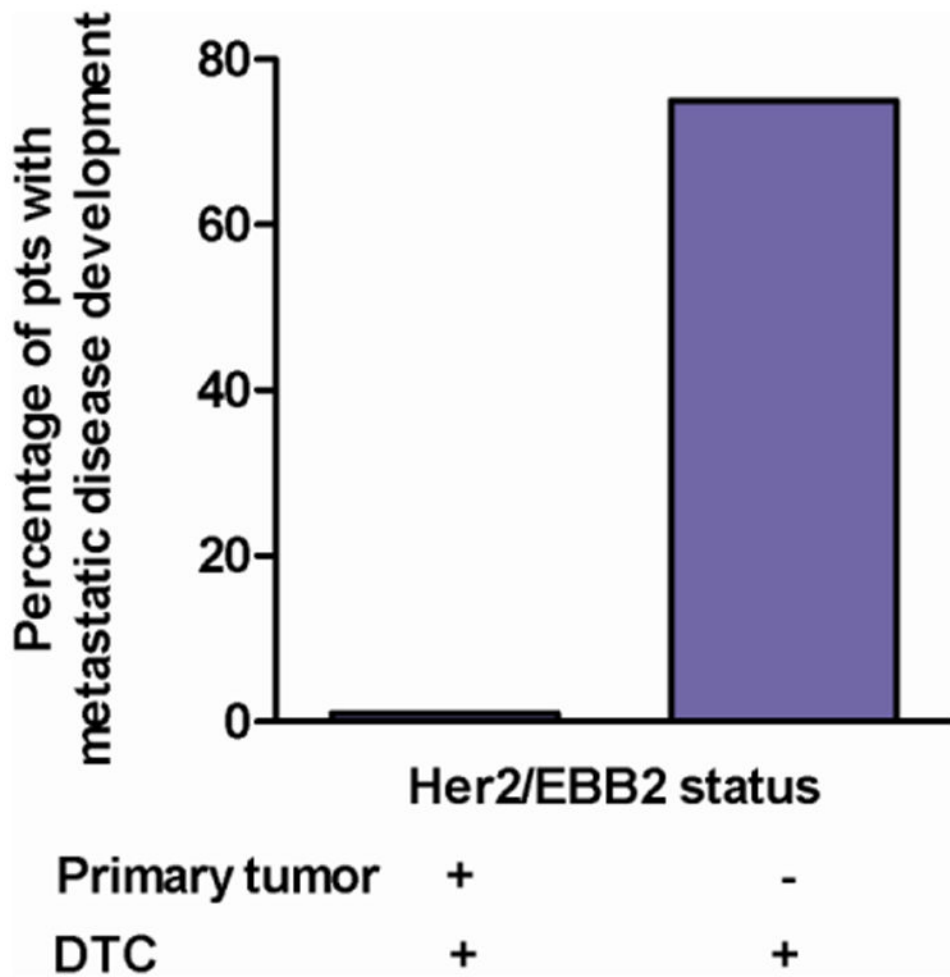
Figure 4.
Recurrent disease development in patients with *ERBB2*-positive DTCs. Patients with Her2 positive tumors received chemotherapy with trastuzumab. Patients with Her2-negative tumors received cytotoxic chemotherapy alone. All patients had *ERBB2*-positive DTCs. All recurrences detected within 24 months (mean=19) of diagnosis.

Table 1

Nanostring nCounter detection of nine DTC–associated transcripts in three different breast tumor cell lines spiked into healthy donor bone marrow at defined cell number dilutions. Numbers indicate total hybridization counts for each transcript in each sample, after subtracting transcript counts from those obtained from healthy bone marrow controls. Transcripts demonstrating detectable expression proportional with dilution of each cell line are indicated in **bold**. Low hybridization counts that are constant despite changing tumor cell dilutions, even after background subtraction, are attributed to signal from non-malignant bone marrow cellular constituents.

Cell Line	SKBR3			MDA-MB231				ZR75			
Dilution	1×10^{-5}	2×10^{-4}	5×10^{-3}	1×10^{-5}	2×10^{-4}	5×10^{-3}	1×10^{-3}	1×10^{-5}	2×10^{-4}	5×10^{-3}	1×10^{-3}
KRT17	-	-	-	-	-	-	-	-	-	-	-
KRT19	**51**	**424**	**1,408**	**-**	**42**	**202**	**1,588**	**90**	**400**	**1,252**	**5,580**
PITX2	-	-	-	-	-	-	-	-	-	-	-
S100A3	13	5	13	**8**	**14**	**25**	**122**	9	3	7	5
SCGB2A2	0	2	5	-	-	-	-	-	**7**	**23**	**112**
SNAI2	4	4	7	**8**	**20**	**49**	**350**	11	3	3	12
EPCAM	38	20	61	9	19	6	32	**3**	**37**	**169**	**761**
TWIST1	10	-	2	-	8	-	3	4	7	11	2
EGFR	7	14	-	**2**	**26**	**108**	**783**	14	25	15	49

Table 2

nCounter signal detection of DTC-associated transcripts in BM specimens from five breast cancer patients with previously documented DTCs by ICC, who also developed metastatic disease. Molecular phenotype and clinical characteristics of the five tumors are shown, including the number of DTCs bone marrow cells detected by immunocytochemistry (ICC) per two million mononucleated cells. Mean signal counts from triplicate assays for each gene transcript in each BM sample are shown with the standard deviation from triplicate samples; values which exceed two standard deviations above the mean of eight bone marrow samples from healthy individuals are in **bold**. Note that *EPCAM* and *SCGB2A2* expression in sample 7547 approached two standard deviations above the reference range, but did not exceed this threshold.

Pt ID	Control BM	2904	9645	9290	7547	6335
ER	--	neg	neg	pos	pos	neg
PR	--	neg	neg	pos	pos	neg
Her-2	--	neg	neg	neg	neg	pos
DTCs by ICC	--	2	4	3	4	10
Months to recurrence	--	15	12	48	15	22
Metastatic site	--	Liver	Lung	Bone	Ovary	Liver, Bone
TWIST1	13 ± 8	8 ± 9	6 ± 2	10 ± 4	2 ± 1	**68 ± 13**
SNAI2	32 ± 40	18 ± 19	**315 ± 10**	66 ± 11	55 ± 9	31 ± 3
EPCAM	293 ± 134	**694 ± 283**	213 ± 13	**446 ± 70**	397 ± 12	178 ± 57
SCGB2A2	5 ± 15	1 ± 1	1 ± 1	1 ± 1	15 ± 5	1 ± 1
EGFR	21 ± 12	11 ± 14	27 ± 3	28 ± 6	17 ± 4	**100 ± 21**
S100A3	32 ± 13	**57 ± 18**	28 ± 1	**78 ± 20**	37 ± 10	38 ± 11
PITX2	10 ± 8	4 ± 2	5 ± 0	10 ± 6	1 ± 2	**105 ± 13**
KRT17	91 ± 48	48 ± 40	**258 ± 25**	101 ± 15	27 ± 7	118 ± 29
KRT19	34 ± 19	22 ± 14	19 ± 5	42 ± 5	9 ± 2	**139 ± 18**