



ORIGINAL ARTICLE

NanoString nCounter® Approach in Breast Cancer: A Comparative Analysis with Quantitative Real-Time Polymerase Chain Reaction, *In Situ* Hybridization, and Immunohistochemistry

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Purpose: Accurate testing for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) is essential for breast cancer treatment. At present, immunohistochemistry (IHC)/fluorescence *in situ* hybridization (FISH) are widely accepted as the standard testing methods. To investigate the value of NanoString nCounter®, we performed its comparative analysis with IHC/FISH and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the assessment of ER, PR, and HER2. **Methods:** Data on IHC/FISH results for ER, PR, and HER2 in 240 patients from a single tertiary hospital in Korea were collected and compared with NanoString nCounter® and qRT-PCR results at a single institution. **Results:** Expression levels for each gene using NanoString nCounter®

showed good correlation with the corresponding data for protein expression by IHC ($p < 0.001$) and gene amplification status for *HER2* ($p < 0.001$). Comparisons between gene expression and IHC data showed good overall agreement with a high area under the curve (AUC) for *ESR1/ER* (AUC = 0.939), *PgR/PR* (AUC = 0.796), and *HER2/HER2* (AUC = 0.989) ($p < 0.001$). **Conclusion:** The quantification of *ER*, *PgR*, and *HER2* mRNA expression with NanoString nCounter® may be a viable alternative to conventional IHC/FISH methods.

Key Words: Breast neoplasms, *ErbB-2*, Gene expression, Immunohistochemistry, *In situ* hybridization

INTRODUCTION

Breast cancer is a biologically heterogeneous disease with diverse natural history and different clinical behaviors. As a result, there has been an increasing shift toward a personalized approach for breast cancer management. To provide the right treatment based on the patient's underlying tumor biology, tumor biomarkers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are routinely used to determine the optimal and most effective therapy. Expression of hormone receptors is observed in approximately 70% of invasive breast can-

cers and acts as a strong predictive biomarker. Patients expressing ER and/or PR may probably benefit from endocrine therapy [1], which is highly effective and less toxic than chemotherapy. Patients with tumors containing as few as 1% invasive cells are known to gain clinical benefits from endocrine therapy [2]. The guidelines of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommend a cutoff of > 1% ER/PR-positive tumor cells to distinguish "positive" from "negative" cases [3]. Therefore, careful examination and accurate assessment of immunohistochemistry (IHC) positivity is absolutely vital to avoid missing potentially significant focal staining.

The *HER2* (also referred to as *ERBB2*) gene, located at chromosome 17q12-21, encodes a transmembrane receptor tyrosine kinase of the human epidermal growth factor receptor family [4] and is overexpressed in approximately 15% to 20% of breast cancers [5]. *HER2* (also called epidermal growth factor receptor *ErbB-2*) signaling pathway promotes cell proliferation and survival, and *HER2* overexpression is associated

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with an aggressive tumor phenotype and reduced survival [6]. Patients with *HER2* amplification usually overexpress *HER2* protein and display worse prognosis than patients with normal *HER2* levels [4,7]. Trastuzumab, a monoclonal antibody against the extracellular domain of *HER2* protein, is the first *HER2*-targeted agent that showed efficacy as a single agent or in combination with chemotherapy in patients with *HER2*-positive invasive breast cancer [8-10]. Other targeted agents such as lapatinib, a reversible dual *HER1/2* tyrosine kinase inhibitor, and pertuzumab were also developed and have improved the prognosis of patients with *HER2*-positive breast cancer [11,12]. In recent years, treatment with trastuzumab has become the standard therapy for patients with *HER2*-positive breast cancer [13] and *HER2* testing is recommended at the time of diagnosis for all breast cancers [14]. Although trastuzumab is effective, it can be toxic in some patients; therefore, accurate assessment of *HER2* overexpression is mandatory to identify patients who may benefit from this targeted therapy.

In 2013, ASCO/CAP recommended two diagnostic methods for the determination of *HER2* status: IHC and *in situ* hybridization (ISH). IHC uses a protein antibody to detect *HER2* protein, whereas ISH employs a DNA probe with a fluorescent, chromogenic, or silver detection system to determine the number of *HER2* gene copies [15,16]. Although the ASCO/CAP guidelines provide relatively clear instructions for the assessment of *HER2* status, inter-laboratory or inter-observer variability may occur owing to the technical issues or difficulty in the interpretation of test results. Interpretations of the results of IHC or ISH for *HER2* status by pathologists may show inter- or intra-observer differences, as both methods involve semi-quantitative or qualitative analyses. Therefore, quantitative methods are needed for the determination of *HER2* expression level to achieve good agreement with the *HER2* status among pathologists.

In this direction, the possibility of using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) as an ancillary test to complement the more subjective methods of IHC and fluorescence *in situ* hybridization (FISH) was raised. Several studies have reported the performance of qRT-PCR to determine *HER2* status [17-19]. These studies concluded that molecular approaches such as qRT-PCR are powerful and reliable quantitative methods for *HER2* status assessment and may complement IHC or FISH for optimal patient treatment. In particular, in the era of personalized molecular target therapy, a high-throughput and cost-effective screening tool such as qRT-PCR becomes even more important for deciding cancer treatment strategies. An optimal assay for molecular studies should be sensitive and specific as well

as easy to perform, readily interpretable, and reliable. qRT-PCR is relatively easy to perform and cost-effective, but the requirement for reverse transcription and PCR amplification may lead to potential error or bias in target gene assessment.

NanoString nCounter® gene expression system is a recently developed RNA-based technology that allows digital quantification of multiplexed target molecules through the use of color-coded barcodes. It can provide discrete counts of target mRNA transcripts using a small amount of total RNA without the need for amplification [20].

This study was performed to assess the usefulness of NanoString nCounter® gene expression system for the quantification of mRNA expression in archived formalin-fixed, paraffin-embedded (FFPE) invasive ductal carcinoma samples as well as to correlate the mRNA expression level of ER, PR, and *HER2* by NanoString nCounter® gene expression system with the results of IHC/FISH and qRT-PCR.

METHODS

Patients and samples

A total of 240 cases of surgically resected invasive breast cancer from 1995 to 2012 were retrospectively retrieved from the computerized records system at Samsung Medical Center. Among them, 45 cases were *HER2*-positive breast cancer patients treated with adjuvant trastuzumab, 143 cases included triple-negative breast cancer, and 52 cases were ER/PR-positive breast cancer. All patients provided written informed consent.

Two pathologists (E.Y.C. and J.H.) reviewed the pathologic findings to determine the following variables: histological subtype, tumor size, nuclear grade, histological grade according to the modified Bloom-Richardson grading system, presence of lymphovascular invasion, and immunohistochemical profiles of ER, PR, Ki-67, and *HER2* without the knowledge of NanoString, qRT-PCR, and *HER2* FISH results.

The Institutional Review Board of Samsung Medical Center, Seoul, Korea approved our study protocol and waived the need for informed consent, as the study was conducted using archival tissues with retrospective clinical data (IRB number: 2017-07-088). All investigations were conducted according to the principles of the Declaration of Helsinki.

Immunohistochemistry and fluorescence *in situ* hybridization

We determined the IHC positivity for ER and PR protein according to ASCO/CAP guidelines of a threshold of 1% and by Allred score [21] using antibodies specific for ER (clone 6F11; Novocastra, Newcastle upon Tyne, UK) and PR (clone 16; Novocastra) [3]. *HER2* status was evaluated using a specific

ic antibody (HercepTest; Dako, Glostrup, Denmark) and/or by FISH. HER2 expression was also defined according to ASCO/CAP guidelines [15]. IHC grades of 0 and 1 for HER2 were defined as a negative result, while grade 3 was recorded as a positive result. Amplification of *HER2* was confirmed using FISH, if a sample was rated 2+ by IHC.

FISH was performed using a dual-color DNA-specific probe kit from PathVision™ (Vysis LSI® HER2 SpectrumOrange™ and CEP17 SpectrumGreen™; Abbott Molecular, Des Plains, USA) in cases with equivocal HER2 IHC results (2+). A total of 50 nuclei per sample were evaluated under a fluorescence microscope (Zeiss Axioskop, Oberkochen, Germany) using filter sets recommended by Vysis (4',6-diamidino-2-phenylindole [DAPI]/Spectrum Orange dual bandpass, DAPI/Spectrum Green dual bandpass). All overlapping nuclei were excluded. The gene copy number ratio was determined according to ASCO/CAP guidelines [15]. After assessment of expression profiling by IHC/FISH, cases were classified into molecular subtypes [22]. In particular, ER or PR+/HER2- tumors with low proliferation index were considered luminal A, high-grade ER or PR+/HER2- tumors and ER or PR+/HER2+ tumors were considered luminal B, whereas ER and PR-/HER2+ tumors were considered HER2-enriched subtype. Triple-negative tumors were considered basal-like [23].

RNA extraction

All available hematoxylin and eosin-stained slides from FFPE primary breast tumor tissue were reviewed. Areas containing representative invasive tumor were carefully marked on the stained slide and microdissected from 2 to 4 paired 4- μ m-thick unstained FFPE sections with a pointed surgical blade. Non-tumor areas were removed by manual microdissection. Total RNA was extracted using the high pure RNA paraffin kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instruction. The yield and purity of RNA was evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, USA). All samples had a total RNA concentration greater than 50 ng/ μ L and were, therefore, included in the analysis.

Real-time quantitative reverse transcription polymerase chain reaction

RNA from archival specimens was measured by real-time qRT-PCR using the TaqMan gene expression assays (Applied Biosystems Inc., Foster City, USA) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an endogenous control (Applied Biosystems; assay ID, HS9999905_m1). RT-PCR was conducted using the high capacity cDNA reverse transcription kit

(Applied Biosystems) according to the manufacturer's instructions. PCR amplification of exons 20 and 21 of *HER2* was performed using the oligonucleotide primers 5'-GGAGCTGAG GAAGGTGAAGGT-3' (forward) and 5'-GATCCAGAT GCCCTTGACTGT-3' (reverse). After incubation for 10 minutes at 95°C, amplification was performed in a thermocycler (Applied Biosystems) with 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 60 seconds, and a final extension at 72°C for 10 minutes. Each complementary DNA sample was analyzed in triplicates with ABI PRISM 7500HT fast real-time PCR (Applied Biosystems). C_T , the fractional cycle number at which the amount of amplified target reached a fixed threshold, was determined and the mRNA expression level of *HER2* was measured using the $2^{-\Delta\Delta C_T}$ ($\Delta\Delta C_T = \Delta C_{\text{target gene}} - \Delta C_{\text{GAPDH}}$) method.

NanoString nCounter® system assays

The nCounter® assay was performed using the NanoString nCounter® Analysis System (NanoString Technologies, Seattle, USA). Hybridization reactions were performed according to the manufacturer's instructions. An nCounter® CodeSet (NanoString Technologies) containing a biotinylated capture probe for target genes and six housekeeping genes (*CLTC*, *GAPDH*, *GUSP*, *HPRT1*, *PGK1*, and *TUBB*) and reporter probes attached to color-barcode tags according to the nCounter® CodeSet design was hybridized to 200 ng of total RNA for 18 hours at 65°C. Samples were processed using an automated nCounter® Prep Station. Hybridized samples were purified and immobilized in a sample cartridge for data collection, followed by the quantification of target mRNA in each sample using the nCounter® Digital Analyzer. For each reaction, 600 fields of view were counted. Quantified expression data were analyzed using nSolver analysis software (NanoString). The resulting counts were normalized to the average counts for all control spikes in each sample. After performing image quality control using a predefined cutoff value, we excluded the outlier samples using a normalization factor based on the sum of positive control counts greater than 3-fold. The counts of the probes were then normalized using the geometric mean of the six housekeeping genes (*CLTC*, *GAPDH*, *GUSP*, *HPRT1*, *PGK1*, and *TUBB*). Data from all 240 patients with breast cancer were included in the statistical analysis.

Statistical analysis

We used the Spearman rank correlation coefficient test for the comparison of (1) *HER2* gene expression level between NanoString and qRT-PCR and (2) *HER2* gene expression level by NanoString and *HER2* average gene copy number by FISH. Jonckheere-Terpstra test and Cohen kappa statistic were used

to compare the NanoString or qRT-PCR results for *HER2* expression with *HER2* IHC results. The correlation between *ESR1* and *PgR* gene expression by NanoString and IHC profiles for ER and PR protein expression was evaluated by the Mann-Whitney test and Cohen kappa statistic. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS software version 18.0 (SPSS Inc., Chicago, USA). To set the cutoff value (low vs. intermediate/high) of the NanoString counts for specific genes (*ESR1*, *PgR*, and *HER2*), receiver operator characteristic (ROC) curves were generated by dichotomizing IHC data. ROC curves were analyzed using SPSS and MedCalc software (MedCalc Software, Mariakerke, Belgium).

RESULTS

Clinicopathological characteristics of 240 breast cancer cases

The general clinicopathological characteristics of 240 breast cancer patients are presented in Table 1. The median age at diagnosis was 46 years (range, 24–74 years). Invasive ductal carcinoma was found in 91.3% of cases and lymph node involvement was present in 42.9% of cases. High histological and nuclear grades were found in 155 patients (64.6%). Of 240 cases, 37 (15.4%) and 17 (7.1%) showed *HER2* positive (3+) and equivocal (2+) results on IHC, respectively. ER-positive and PR-positive tumors were noted in 57 (23.8%) and 53 (22.1%) cases, respectively. According to the results of IHC/FISH profile for 240 cases, 48 cases were ER or PR(+)/*HER2*(-), 21 cases were ER or PR(+)/*HER2*(+), 24 cases were ER or PR(-)/*HER2*(+), and 143 cases were ER or PR(-)/*HER2*(-). Two of 240 cases were ER or PR(-) but showed *HER2* equivocal results in both IHC and FISH. Two of 240 cases were ER or PR(+) but failed to show adequate results for *HER2* FISH.

Comparison between NanoString and IHC/FISH for ER, PR, and *HER2* expression

To evaluate the concordance of NanoString nCounter® and conventional methods for determining the molecular subgroup of breast cancer, we compared the NanoString gene expression level for *ESR1*, *PgR*, and *HER2* with IHC results for ER, PR, and *HER2* proteins and FISH results for *HER2* gene. The expression level of each gene using NanoString nCounter® showed a good correlation with levels of the corresponding protein biomarkers by IHC ($p < 0.001$) (Figure 1).

The samples with ER-negative results on IHC showed nCounter® levels from 5.03 to 491.01 (median, 28.11), while those with ER-positive results on IHC displayed nCounter® levels from 10.58 to 4,594.15 (median, 426.8).

The samples with PR-negative results on IHC showed

Table 1. Clinicopathologic characteristics of 240 specimens

Characteristic	No. of patients (%)
Age at the surgery (yr)*	46 (24–74)
Tumor size (cm)	
≤2	92 (38.3)
>2, ≤5	143 (59.6)
>5	5 (2.1)
Tumor type	
Ductal	220 (91.3)
Lobular	2 (0.8)
Others	18 (7.5)
Lymph node status	
Negative	137 (57.1)
Isolated tumor cells	1 (0.4)
1–3 node positive	67 (27.9)
4–9 node positive	21 (8.8)
≥10 node positive	14 (5.8)
Histological grade	
1	4 (1.7)
2	68 (28.3)
3	155 (64.6)
Not applicable	13 (5.4)
ER status (IHC)	
Positive	57 (23.8)
Negative	183 (76.3)
PR status (IHC)	
Positive	53 (22.1)
Negative	187 (77.9)
<i>HER2</i> status (IHC)	
0	152 (63.3)
1+	34 (14.2)
Equivocal (2+)	17 (7.1)
Positive (3+)	37 (15.4)
<i>HER2</i> status (FISH, n=25)	
Inadequate	2 (8.0)
Negative	9 (36.0)
Equivocal	3 (12.0)
Positive	11 (44.0)

ER=estrogen receptor; IHC=immunohistochemistry; PR=progesterone receptor; *HER2*=human epidermal growth factor receptor 2; FISH=fluorescence *in situ* hybridization.

*Median (range).

nCounter® levels from 2.35 to 219.32 (median, 18.14), while those with PR-positive results on IHC displayed nCounter® levels from 5.66 to 4,190.71 (median, 74.02). A statistically significant difference was observed between mean nCounter® levels of negative and positive ER/PR IHC groups ($p < 0.001$) (Figure 1A and 1D, 1B and 1E, respectively).

The samples with *HER2* IHC score 0 showed nCounter® levels from 147.18 to 2,302.25 (median, 508.97) and the samples with *HER2* IHC score 1 showed nCounter® levels from 306.42 to 4,293.16 (median, 1,077.85). The equivocal cases with *HER2* IHC (2+) had nCounter® levels ranging from 1,207.76 to 10,127.69 (median, 1,948.67), while the positive

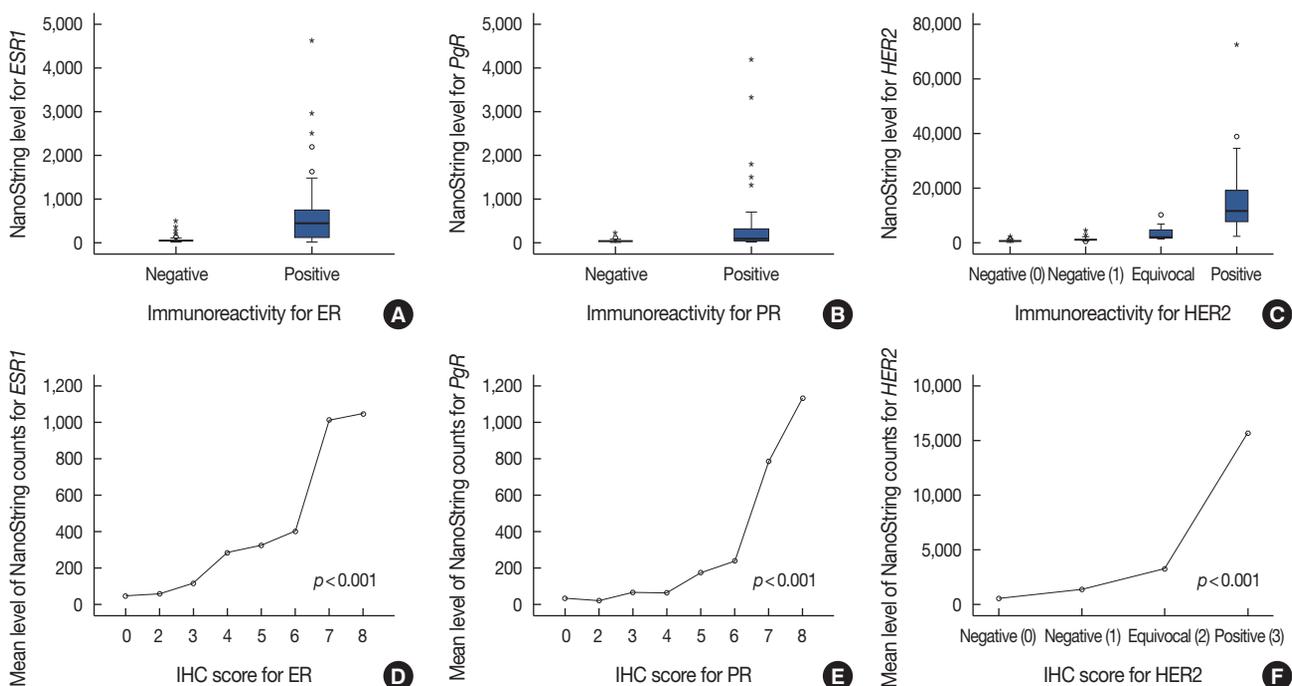


Figure 1. Gene expression levels for *ESR1*, *PgR*, and *HER2* by NanoString in comparison with corresponding protein biomarkers (immunohistochemistry [IHC]). Gene expression levels for *ESR1* (A, D), *PgR* (B, E), and *HER2* (C, F) using NanoString nCounter[®] had good correlation with the corresponding protein biomarkers (IHC) ($p < 0.001$).

ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor 2.

cases with HER2 IHC (3+) showed nCounter[®] levels from 2,229.67 to 72,436.31 (median, 11,666.14). A statistically significant difference was observed between mean nCounter[®] levels of negative, equivocal, and positive HER2 IHC groups ($p < 0.001$) (Figure 1C, F).

The gene expression level for *HER2* using NanoString nCounter[®] showed a good correlation with the gene amplification status for *HER2* ($p < 0.001$) (Figure 2A-C).

Comparison between *HER2* gene expression by qRT-PCR and *HER2* expression by IHC/FISH

We evaluated the correlation between *HER2* gene expression level by qRT-PCR, *HER2* IHC results, and FISH data. The gene expression level (relative quantitation [RQ]) for *HER2* using qRT-PCR showed a good correlation with *HER2* protein expression results with IHC ($p < 0.001$, box plot not shown) but not with *HER2* gene amplification status with FISH ($p = 0.124$) (Figure 2D-F).

Estimation of cutoff values for gene expression using ROC curves

Comparisons between gene expression and IHC data showed good overall agreement with a high area under the curve (AUC) for *ESR1/ER* (AUC = 0.939), *PgR/PR* (AUC =

0.796), and *HER2/HER2* (AUC = 0.989) ($p < 0.001$). The cutoff values of each gene, estimated with consideration of sensitivity and specificity for the detection of specific genes, are shown in each graph (Figure 3).

Concordance of NanoString level and IHC/FISH expression

We compared the results between NanoString level and IHC/FISH for ER, PR, and *HER2* expression (Table 2). Using NanoString cutoff for *ER* and *PgR* expression, we found that the concordance rate of ER and PR expression status by IHC and NanoString method was 82.1% and 79.6%, respectively (Cohen kappa = 0.596 and 0.461, respectively).

Using NanoString cutoff for *HER2* expression, we found that the concordance rate of *HER2* expression status by IHC and NanoString method was 94.2% when we excluded the cases with equivocal *HER2* expression results for IHC (Cohen kappa = 0.833).

Comparison between the results of IHC, FISH, and NanoString counts for *HER2*

The general distribution of *HER2* for 240 specimens is presented in Figure 4. FISH analysis was performed for 25 cases, including all 17 equivocal cases by IHC and eight controls that had relatively ambiguous NanoString counts in comparison

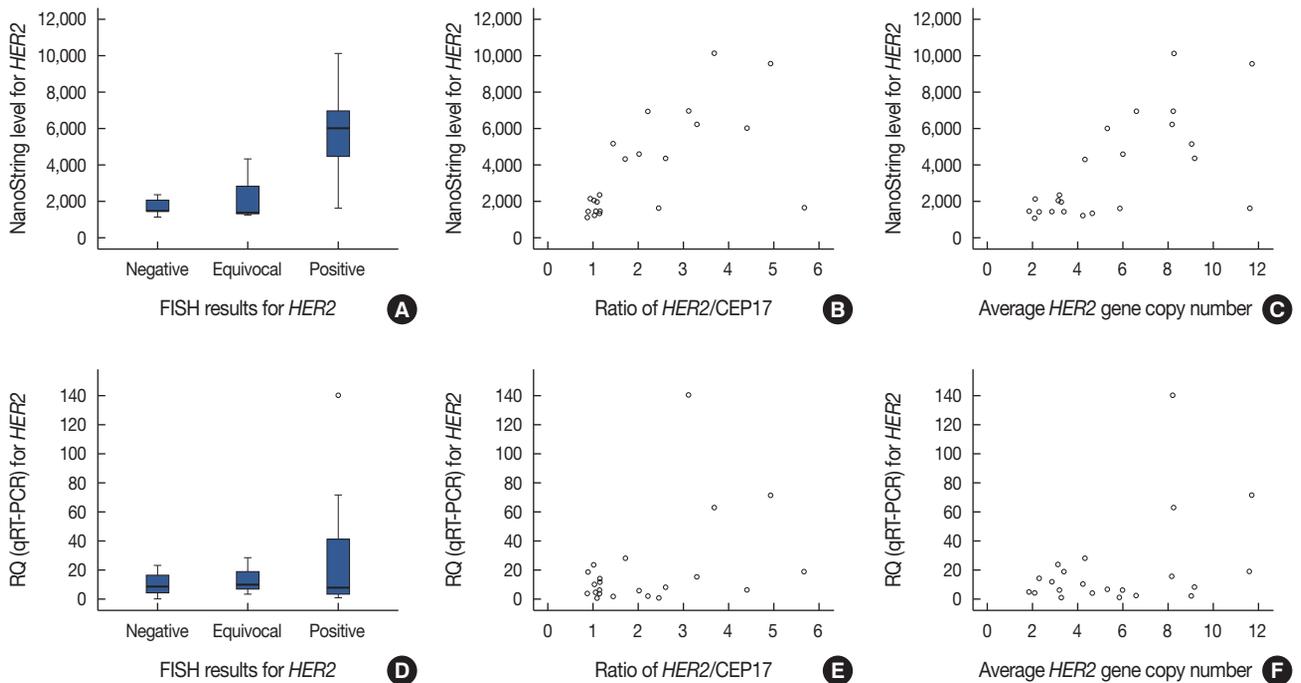


Figure 2. Gene expression levels for *HER2* by NanoString (A-C) and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) (D-F) in comparison with the gene amplification status by fluorescence *in situ* hybridization (FISH). Gene expression levels for *HER2* using NanoString nCounter® had good correlation with the gene amplification status by FISH ($p < 0.001$). *HER2* = human epidermal growth factor receptor 2; RQ = relative quantitation.

with other cases. Adequate results were obtained for 23 cases. Two of the equivocal cases failed to produce appropriate FISH results due to poor sample condition.

The concordance rate of *HER2* expression status by IHC and NanoString method was 94.2% and the discordance rate was 5.8% when we excluded the cases with equivocal *HER2* expression results for IHC.

One case showed *HER2* negativity (1+) in invasive cancer but *HER2* positivity in ductal carcinoma *in situ* (DCIS) by IHC. In the invasive area, FISH result was equivocal and the NanoString count was 4,293.16.

Among the 17 cases that were equivocal for *HER2* by IHC, five showed negative FISH results and their median NanoString count was 1,948.67 (range, 1,425.16–2,330.70). All these cases were considered positive by NanoString level. Two of the 17 cases showed equivocal results by FISH and their median NanoString count was 1,266.14 (range, 1,207.76–1,324.52). All these cases were considered negative by NanoString level. Eight of the 17 equivocal *HER2* cases by IHC showed positive FISH results and had a median NanoString count of 4,859.76 (range, 1,589.55–10,127.69). All these cases were considered positive by NanoString level. Two cases of equivocal *HER2* by IHC failed to yield adequate FISH results. Therefore, seven of

Table 2. Concordance of NanoString level and IHC/FISH expression

IHC/FISH	NanoString		Total No. (%)
	Negative, No (%)	Positive, No (%)	
ER IHC			
Negative	143 (59.6)	40 (16.7)	183 (76.3)
Positive	3 (1.3)	54 (22.5)	57 (23.8)
Total	146 (60.8)	94 (39.2)	240 (100)
PR IHC			
Negative	155 (64.6)	32 (13.3)	187 (77.9)
Positive	17 (7.1)	36 (15.0)	53 (22.1)
Total	172 (71.7)	68 (28.3)	240 (100)
HER2 IHC (score)			
Negative (0)	147 (61.3)	5 (2.1)	152 (63.3)
Negative (1)	26 (10.8)	8 (3.3)	34 (14.2)
Equivocal (2)	2 (0.8)	15 (6.3)	17 (7.1)
Positive (3)	0	37 (15.4)	37 (15.4)
Total	175 (72.1)	65 (27.1)	240 (100)
HER2 FISH			
Inadequate	0	2 (8.0)	2 (8.0)
Negative	1 (4.0)	8 (32.0)	9 (36.0)
Equivocal	2 (8.0)	1 (4.0)	3 (12.0)
Positive	0	11 (44.0)	11 (44.0)
Total	3 (12.0)	22 (88.0)	25 (100)

IHC = immunohistochemistry; FISH = fluorescence *in situ* hybridization; ER = estrogen receptor; PR = progesterone receptor; *HER2* = human epidermal growth factor receptor 2.

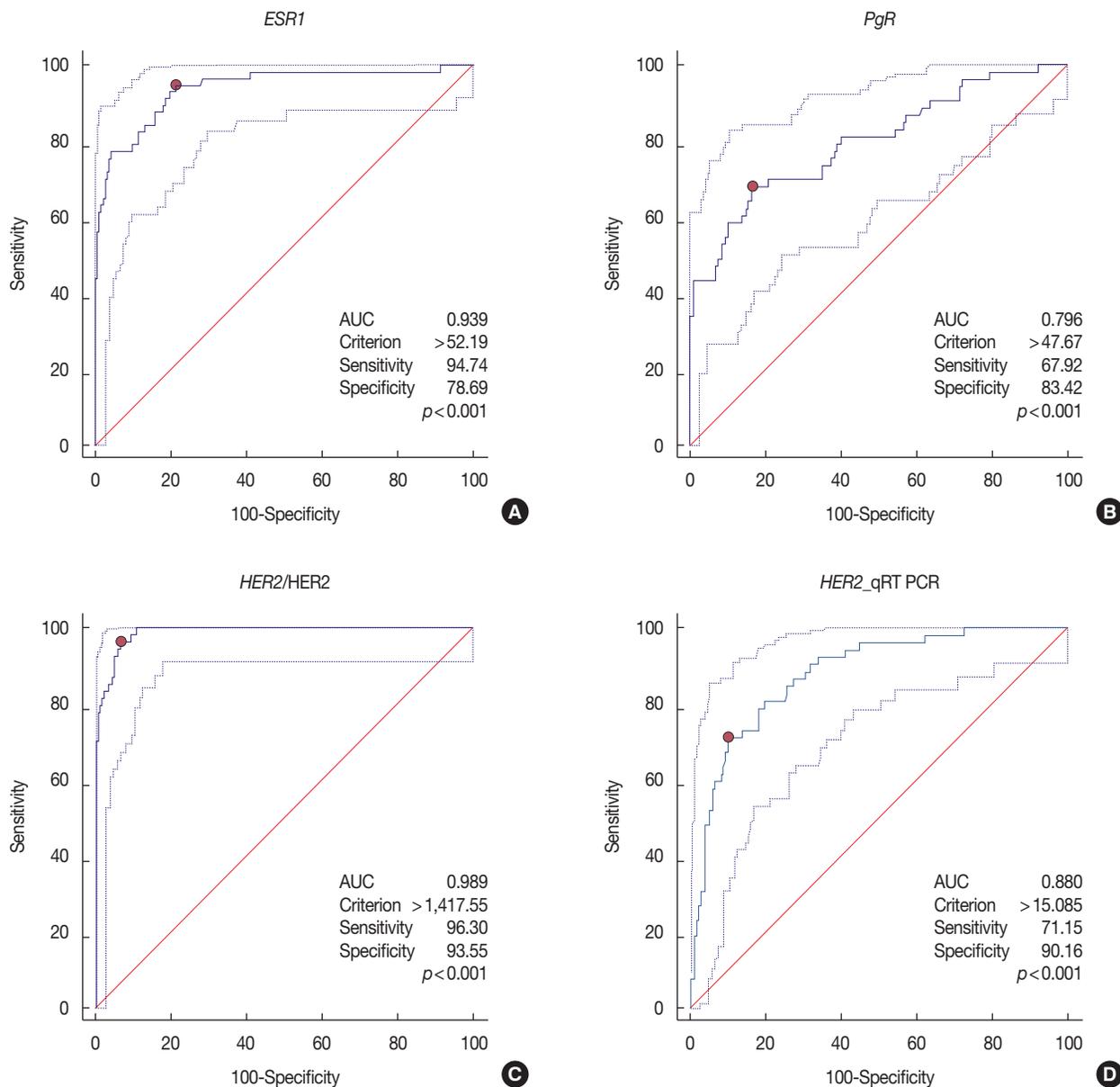


Figure 3. The estimation of overall agreements for gene expression levels compared with immunohistochemistry (IHC) results by receiver operator characteristic curve. Comparisons between the gene expression by NanoString and IHC data gave good overall agreement with a high area under the curve (AUC) for *ESR1/ER* (AUC=0.939) (A), *PgR/PR* (AUC=0.796) (B), and *HER2/HER2* (AUC=0.989) (C) ($p < 0.001$, respectively). The comparison between the gene expression by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and IHC data gave also good overall agreement with a high AUC for *HER2/HER2* (AUC=0.880) (D) ($p < 0.001$).

ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor 2.

15 cases (46.7%) that were HER2 equivocal by IHC showed discrepancy between NanoString and FISH results.

Of the 15 HER2 equivocal cases by IHC that had FISH results, seven cases (46.7%) revealed an elevated CEP17 count, indicative of polysomy. Five of these seven cases that showed FISH negative/equivocal results had an elevated CEP17 count. The median *HER2* and CEP17 copy numbers of FISH-nega-

tive cases ($n=5$) were 3.26 (range, 2.84–3.38) and 3.1 (range, 2.46–3.78), respectively, while the median *HER2* copy number of FISH-equivocal cases ($n=2$) was 4.43 (range, 4.22–4.64). All cases of CEP17 copy number of FISH-equivocal cases were 4.08. Among the eight HER2 equivocal cases on IHC with FISH-positive results, two showed elevated CEP17 count with median *HER2* and CEP17 copy numbers of 9.09 (range,

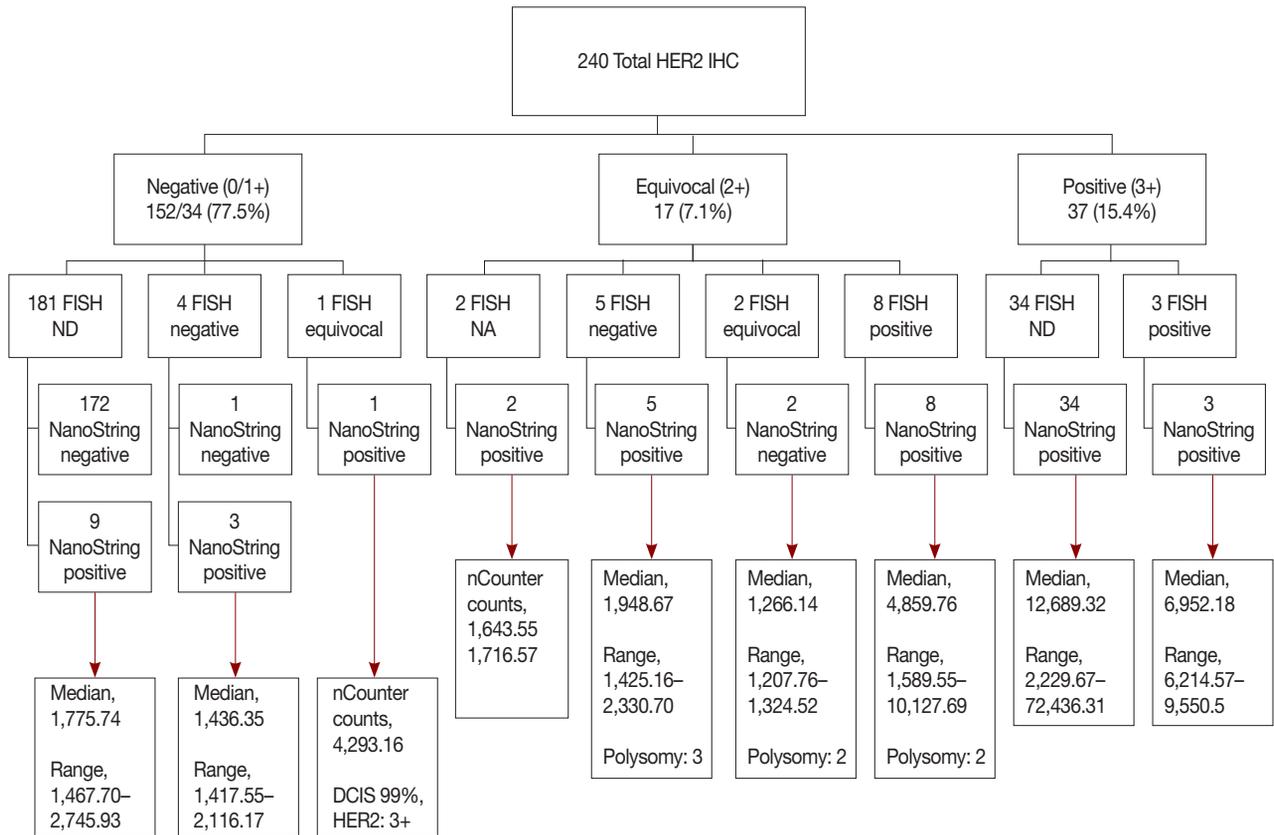


Figure 4. The general distribution of HER2 results of 240 specimens by FISH, IHC, and NanoString. HER2=human epidermal growth factor receptor 2; IHC=immunohistochemistry; FISH=fluorescence *in situ* hybridization; ND=not done; NA= data not applicable due to test failure; DCIS=ductal carcinoma *in situ*.

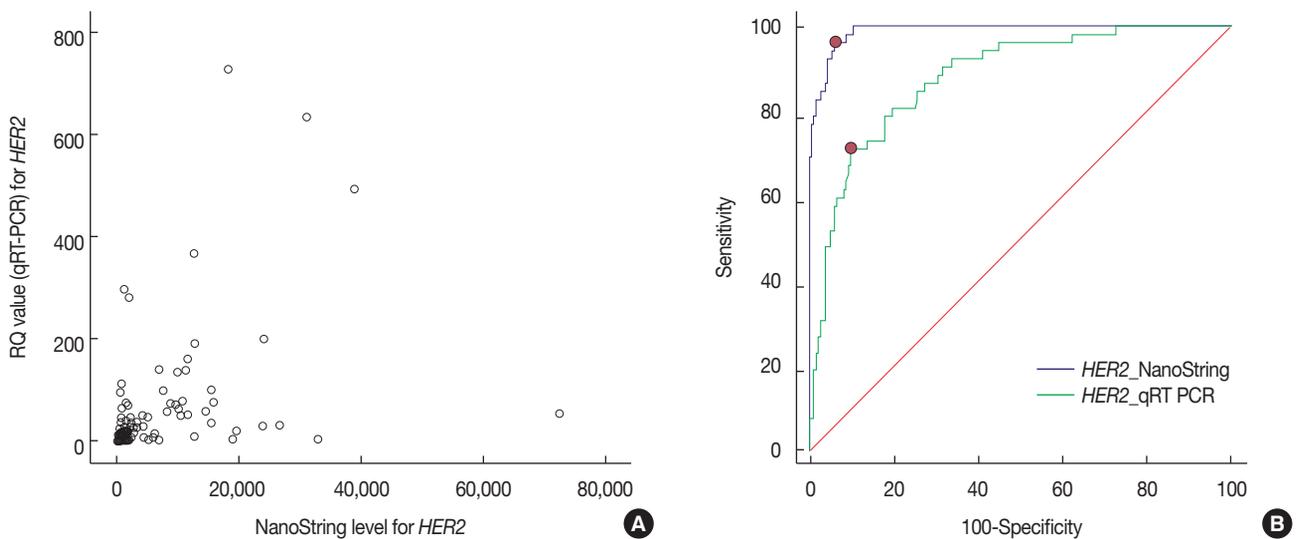


Figure 5. Correlation with NanoString counts for *HER2* gene and relative quantitation (RQ) levels generated by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). (A) NanoString counts for *HER2* gene showed high correlation with RQ levels generated by qRT-PCR ($p < 0.001$). (B) Area under the curve of NanoString method is bigger than that of qRT-PCR method. The pairwise comparison of two receiver operator characteristic curves showed the statistically significant difference ($p < 0.001$). HER2=human epidermal growth factor receptor 2.

5.30–11.60) and 4.89 (range, 1.20–6.26), respectively.

Comparison of NanoString and qRT-PCR for *HER2* gene expression

We performed comparison between the sensitivity and specificity of NanoString nCounter[®] and qRT-PCR for gene expression level. NanoString counts for the *HER2* gene showed high correlation with RQ levels generated by qRT-PCR ($p < 0.001$) (Figure 5A). Although both methods showed good overall agreement between gene expression and IHC data with a high AUC for *HER2/HER2* (AUC of 0.989 for NanoString and 0.880 for qRT-PCR; $p < 0.001$ for each method), the AUC of the NanoString method was greater than that of qRT-PCR. Pairwise comparison of the two ROC curves showed a statistically significant difference ($p < 0.001$) (Figure 5B). As AUC is a measure of the overall value of a diagnostic test, these data indicate that NanoString has better overall performance as a diagnostic test than qRT-PCR.

DISCUSSION

In this study, we described an ancillary method for the detection of the molecular status of invasive breast cancer using NanoString-based gene expression technology. This method relies on the direct digital detection of gene expression. The main goal of this study was to compare the results of NanoString-based gene expression with those of the conventional IHC/FISH methods for the assessment of protein/mRNA expression. We specifically focused on the three most common molecular markers in breast cancer: ER, PR, and HER2. These three biomarkers are regarded as targets for specific treatment in breast cancer and have important value in molecular classification with respect to the survival rate of patients with breast cancer [23].

At present, the combination of IHC/FISH is routinely used for the detection of ER, PR, and HER2 status. We evaluated the accuracy of NanoString-based assay by comparing it with the conventional biomarker detection method. The sensitivity and specificity of NanoString-based assay as compared with that of IHC was 94.7% vs. 78.7% for ER, 67.9% vs. 83.4% for PR, and 96.3% vs. 93.6% for HER2. Although we have compared these methods using only three biomarkers, the sensitivity and specificity of NanoString-based assay appears to be significantly reliable [24,25]. However, some discrepancies were reported in the ER/PR protein expression status by IHC and the gene expression status by NanoString, which may be associated with the presence of the normal breast tissue adjacent to the tumor. Therefore, careful microdissection of invasive cancer is essential for the evaluation of *ER/PgR* gene status

using NanoString method. The discordance rate of HER2 level was low, as HER2 protein overexpression was found only in tumor tissues. Some discrepancy in HER2 protein expression and gene expression was also observed in a relatively small number of cases. A majority of discordant cases were HER2 equivocal by IHC/FISH. One of them contained approximately 1% HER2 negative (1+) invasive cancer and approximately 99% HER2 (3+) DCIS. FISH result was equivocal in the invasive area. Some of the HER2-positive DCIS components may have been included in the microdissected samples of invasive carcinoma in the course of the experiment. With the exception of HER2 equivocal cases by IHC/FISH, 12 out of 240 cases were inconsistent and IHC/FISH (-)/NanoString (+). This observation may be related to the technical factors associated with the experiment and interpretation of raw data [25].

Of the 15 HER2 equivocal cases by IHC that had FISH results, elevated CEP17 count (polysomy) was observed in five of seven cases that showed FISH-negative/equivocal results and two of eight cases with FISH-positive results. Although the clinical implications of elevated CEP17 count (polysomy) are ambiguous, some studies have linked elevated CEP17 count with adverse clinical features and HER2 overexpression [26,27]. In addition, studies have shown that elevated CEP17 copy number may account for trastuzumab response in tumors with a normal *HER2:CEP17* ratio [28,29]. This is the reason some pathologists suggest the use of mean *HER2* copy number rather than *HER2:CEP17* ratio to determine HER2 status in presence of CEP17 co-amplification [16]. According to this recommendation, the absolute quantification of *HER2* gene expression is very helpful for the determination of HER2 status. A recent study [30] reported an association between high levels of HER2 and achievement of a pathologic complete response with neoadjuvant treatment. This suggests that accurate measurement of HER2 may predict the likelihood of response in the presurgical setting. In this direction, NanoString assay represents a strong alternative tool to evaluate the HER2 status, as it is quantitative, reproducible, and easy to perform. Many studies have focused on the quantification of *HER2* expression by qRT-PCR and there is a good correlation between qRT-PCR and conventional IHC/FISH methods [18]. However, AUC of NanoString method was observed to be greater than that of qRT-PCR in our study. As AUC is a measure of the overall value of a diagnostic test, our data suggest that NanoString method displays better overall performance as a diagnostic test than qRT-PCR. In addition, qRT-PCR is more sensitive to RNA quality and requires a gene amplification process, which may be a cause of error in the determination of gene status.

Until recent years, oncologists would require an appropriate tumor specimen to detect various potential molecular markers for personalized cancer treatment. Thus, there is a need for an efficient biomarker profiling assay that can be performed with a limited tumor sample. Hence, high-throughput gene screening methods such as NanoString-based assay are useful for comprehensive cancer genome study of individual patient. In particular, NanoString method can profile 250 genes associated with breast cancer using only 200 ng of total RNA. Moreover, NanoString assay has outstandingly high sensitivity, reproducibility, and a wide dynamic range [20].

Although, NanoString-based assay offers several advantages, there are some limitations associated with this method. In comparison to IHC/FISH or qRT-PCR, this technology is relatively expensive mainly due to the acquisition and maintenance of devices. However, this method can be considered as cost-effective owing to its ability to analyze a variety of genes at once with a limited sample. Aside from its high cost, it is difficult for this method to present the delicate tumor characteristics with genetic complexity of tumor, including intratumoral heterogeneity, copy number variations (polysomy), structural alteration in the target gene, and structure and function of proteins predicted to be altered by genetic alteration [25]. However, this method may be useful by specifically focusing on the expression levels of most commonly screened, targetable markers such as ER, PR, and HER2 in breast cancer.

Our results revealed high concordance between NanoString-based gene assay and IHC/FISH test for ER, PR, and HER2 in breast cancer. Therefore, NanoString-based assay may be a feasible screening tool for initial identification of breast cancer patients that can undergo treatment with specific targeted agents.

In conclusion, our results suggest a high concordance between IHC assessment of ER, PR, and HER2 and NanoString-based multigene assay. The NanoString-based multigene assay showed high specificity and sensitivity in the evaluation of the molecular status of breast cancer. Based on our study, the comprehensive NanoString-based panel of 250 genes may be a useful and efficient high-throughput screening tool for the identification of genetic alterations of potential therapeutic targets, including ER, PR, and HER2, in the era of personalized medicine.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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