Original Article

Evaluation of BrightGen HR RT-qDx assay to detect nuclear receptors mRNA overexpression in FFPE breast cancer tissue samples for selection of tamoxifen therapy

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Abstract: Breast cancer is a significant cause of death in women. Estrogen receptor (ER) and progesterone receptor (PR) are important prognostic factors indicating higher recovery rate in the breast cancer patients. Currently, immunohistochemical (IHC) staining is a conventional method to identify expression of ER and PR. If a breast cancer patient expresses ER or PR, a chemotherapy with estrogen inhibitors such as tamoxifen is supposed to be effective. Although IHC staining is a reliable method, it may not a useful method for continuous monitoring of ER and PR expression changes in multiple breast cancer patients. In the present study, we evaluated an alternative method of IHC for detection of ER and PR expression. A quantitative RT-PCR method called 'the BrightGen HR RT-qDx assay' was employed to detect mRNA expression of the nuclear receptors in 199 formalin-fixed paraffin-embedded (FFPE) breast cancer tissue samples. Among the ER/PR positive samples by IHC, 83 were determined positive and 16 were determined negative for the nuclear receptor mRNA by the quantitative RT-PCR method. Among the ER/PR negative samples by IHC, 37 were determined negative and 2 were determined positive by the quantitative RT-PCR method. The overall sensitivity and specificity of the quantitative RT-PCR method were 83.8% and 94.8% (*P* = 0.0026), respectively. We also optimized the quantitative RT-PCR method by setting up the diagnostic cut-off value using the likelihood ratio. The highest likelihood ratio was when the expression levels of the relative nuclear receptor mRNA passed 103.3 at which sensitivity and specificity was highest. These data suggest that BrightGen HR RT-qDx assay could be an alternative method for detection of the prognostic factors of nuclear receptors expressed in breast cancer patients for providing essential information for therapeutic application of tamoxifen.

Keywords: Breast cancer, FFPE, ER, PR, RT-qPCR, molecular diagnosis

Introduction

Breast cancer is the most diagnosed and the second leading cause of cancer deaths for women, occurring in about 300,000 and killing about 40,000 women a year [1]. A substantial body of epidemiological, experimental and clinical evidence has indicated that treatment of breast cancer epithelial cells with estrogen induced progression of breast cancers [2].

Breast cancer is often considered a systemic disease because even the early tumor cells disseminate [2]. Therefore, pathological outcome of breast cancer is largely dependent on the metastases of early tumor cells. Estrogen receptor (ER) and progesterone receptor (PR) are regarded as prognostic and predictive factors of breast cancer [3]. ER and PR are members of nuclear receptors, of which activities are regulated by small hydrophobic molecules

Table 1. Relative NR mRNA expression levels detected by NR mRNA RT-qPCR in various cell lines

Cell line	NR (ER/PR) status	Origin of cell	Relative NR mRNA expression level
SK-BR-3	Weak-expressing	Breast cancer	3.66 ± 1.34
MCF-7	Over-expressing	Breast cancer	92.78 ± 7.42
MDA-MB-231	Weak-expressing	Breast cancer	1
THP-1	Over-expressing	Monocyte	52.16 ± 4.2.

agonist and antagonist [4]. ER regulates expression of a variety of genes involved in cell growth, so that it plays a critical role in the growth of breast cancer [4]. Because of that reasons, expression of the ER has become an important biomarker for pathogenesis and maintenance of breast cancer. Approximately 70% of breast tumors express the ER [5, 6] and endocrine therapies, such as the anti-estrogen tamoxifen, are the most common and effective therapies for patients with ER-positive breast cancer. Thus, ER is the single most important prognostic and predictive factor determining treatment outcomes [7, 8]. PR also plays significant roles in breast cancer biology. PR, an estrogen-regulated gene, is often co-expressed with ER, and independently predicts breast cancer outcomes, and has been implicated in regulating stem-like cancer cells [9].

Tamoxifen, raloxifene, and fulvestrant are selective ER modulators (SERMs) that are important therapeutic agents for women with endocrine-sensitive (ER-positive) breast cancer because SERMs can effectively antagonize the proliferation-inducing effects of estrogen. The estrogen effect also can be antagonized by inhibitors of aromatase, which is an enzyme for estrogen generation, such as anastrozole and letrozole. The detection of ER and PR by immunohistochemistry (IHC) is not a decisive prognostic marker for breast cancer, but it is essential marker indicating susceptibility for endocrine therapy with SERMs [10]. To detect ER and PR in cancer tissues, IHC has been widely used [11]. IHC gives robust signals for many biomarkers, however, the intensity of IHC depends on the enzyme activity of horseradish peroxidase (HRP), which is conjugated on antibodies. Therefore, the staining intensity is significantly influenced by reaction time, temperature, and HRP substrate concentrations which are often vary depending on the extent of training of experimenters. Despite the international efforts to standardize the cut-offs and the method of IHC staining, a complete standardization for assessment of ER and PR with IHC is still challenging owing to the inherent semi-quantitative nature of the technology [12, 13].

As an alternative of the IHC assay, a quantitative RT-PCR method targeting mRNA of ER and PR has been proposed [13]. In the present study, the quantitative RT-PCR method was employed for quantification of ER and PR expression in 199 formalin-fixed paraffin-embedded (FFPE) tissue samples from breast cancer patients. We used a commercial kit called 'BrightGen HR RT-qDx' that uses one-tube nested RT-PCR process. Through the experiments, we evaluated whether the quantitative RT-PCR (RT-qPCR) method can be a reliable alternative method of current IHC method for detection of ER and PR in clinical tissue samples.

Materials and methods

Clinical samples

For this study, we selected a total of 199 formalin-fixed paraffin-embedded (FFPE) tissue samples obtained from patients diagnosed breast cancer at Yonsei University Severance Hospital (Seoul, Republic of Korea) for 2 years. These tissue samples have been confirmed the expression of ER, PR, and HER2 with IHC method. All subjects were provided with a written informed consent and the study was approved by the Institutional Ethics Committee of Yonsei University Severance Hospital (approval number 1-2010-0018).

Cell lines and cell culture

Human breast carcinoma cell line MCF7, which over-expresses ER and PR, and MDA-MB-231, which weakly expresses ER and PR, were kindly provided by the Yonsei University Cancer Center (Seoul, Republic of Korea). The THP-1 human monocytic cell line, which over-expresses ER, was kindly provided by Yonsei University College of Medicine (Seoul, Republic of Korea). The SK-BR3, MCF7, and MDA-MB-231 cancer cells were maintained as mono-layer cultures in DMEM at 37°C, 5% CO_2. THP-1 cells were cultured in RPMI 1640 supplemented with 2 mM

A

B

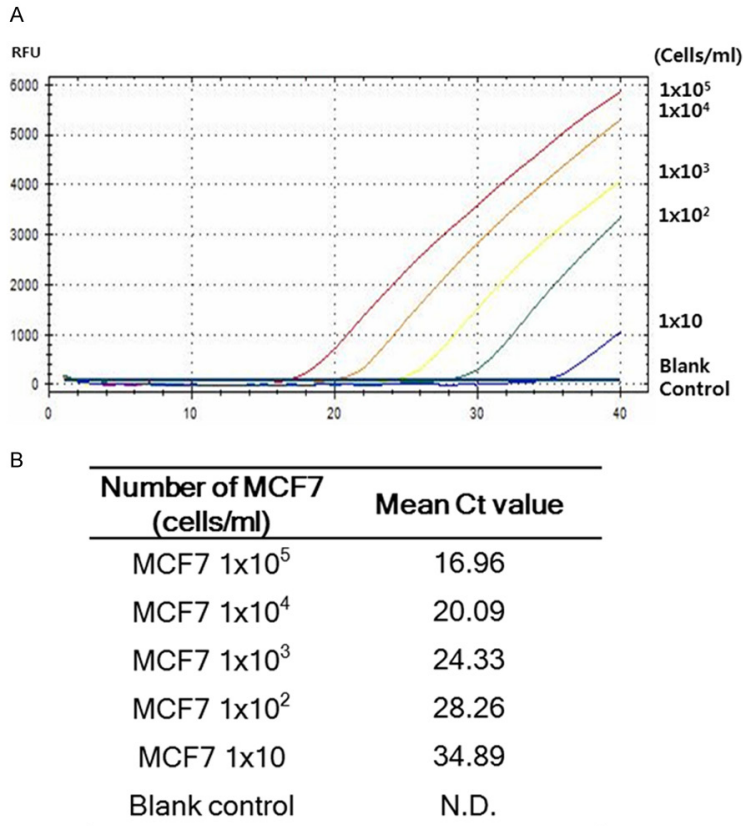| Number of MCF7 (cells/ml)	Mean Ct value
MCF7 1×10^5	16.96
MCF7 1×10^4	20.09
MCF7 1×10^3	24.33
MCF7 1×10^2	28.26
MCF7 1×10	34.89
Blank control	N.D.

Figure 1. The analytical sensitivity of HR mRNA RT-qPCR. A. The analytical sensitivity of HR mRNA RT-qPCR was determined using serially diluted breast cancer cell line MCF7, an ER and PR over-expressing cell line. B. HR mRNA RT-qPCR was performed three times and the mean Ct value was calculated (N.D.: Not detected).

glutamine. All of the media was supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Gibco-BRL, Carlsbad, California, USA).

Deparaffinization and RNA isolation from the FFPE breast cancer tissues

In order to remove paraffin from FFPE tissue samples, two pieces of 10-µm-thick FFPE breast cancer tissue sections were put in a 1.5-mL microcentrifuge tube, to which 1 mL of 100% xylene was added. After shaking and vortexing, the tube was heated for 5 min at 50°C to melt the paraffin and centrifuged for 2 min at room temperature at 20,000 x g to precipitate the tissue. The xylene supernatant was removed, and 1 mL of 100% EtOH was added to the pellet. After mix with EtOH, the tube was centrifuged at 20,000 x g for 2 min at room temperature, and then the EtOH was removed

without disturbing the pellet. The ethanol washing repeated twice. Residual EtOH was removed as much as possible without disturbing the pellet, and the pellet was dried in the air for 25 min.

A total RNA isolation kit, 'MagNA Pure LC RNA Isolation Kit III-Tissue' (Roche Diagnostics, Mannheim and Penzberg, Germany), was used according to the manufacturer's protocol for total RNA extraction. In brief, 140 µL of tissue homogenized buffer (Roche Diagnostics, Ma-nnheim and Penzberg, Germa-ny) and 16 µL of 10% SDS solution were added to the deparaffinized tissue, sequentially. Then, the mixed samples were vortexed and incubated overnight at 55°C, after which 220 µL of tissue lysis buffer (Roche Diagnostics) was added to the tissue lysate supernatant. Then, Mag-NA Pure LC 2.0 (Roche Diagnostics, Mannheim and Penzberg, Germany) machine was used to prepare total RNA. The purity and concentration of total RNA were determined by measuring the absorbance at 260 nm and 280 nm using the Infinite 200® spectrophotometer (Tecan, Salzburg, Austria). All steps in the preparation and handling of total RNA was conducted in a laminar flow hood under RNase-free conditions. The isolated total RNA was stored at -70°C until used for cDNA synthesis.

cDNA synthesis

Complementary DNA (cDNA) was synthesized using an M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random hexamers (Invitrogen) according to the manufacturer's protocols. Briefly, 10 µL of total RNA was added to the master mix containing 1 µL of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, and dTTP at a neutral pH), 0.25 ug of random hexamers, and 5 µL of DEPC-treated water in a PCR tube. The reaction mixture was incu-
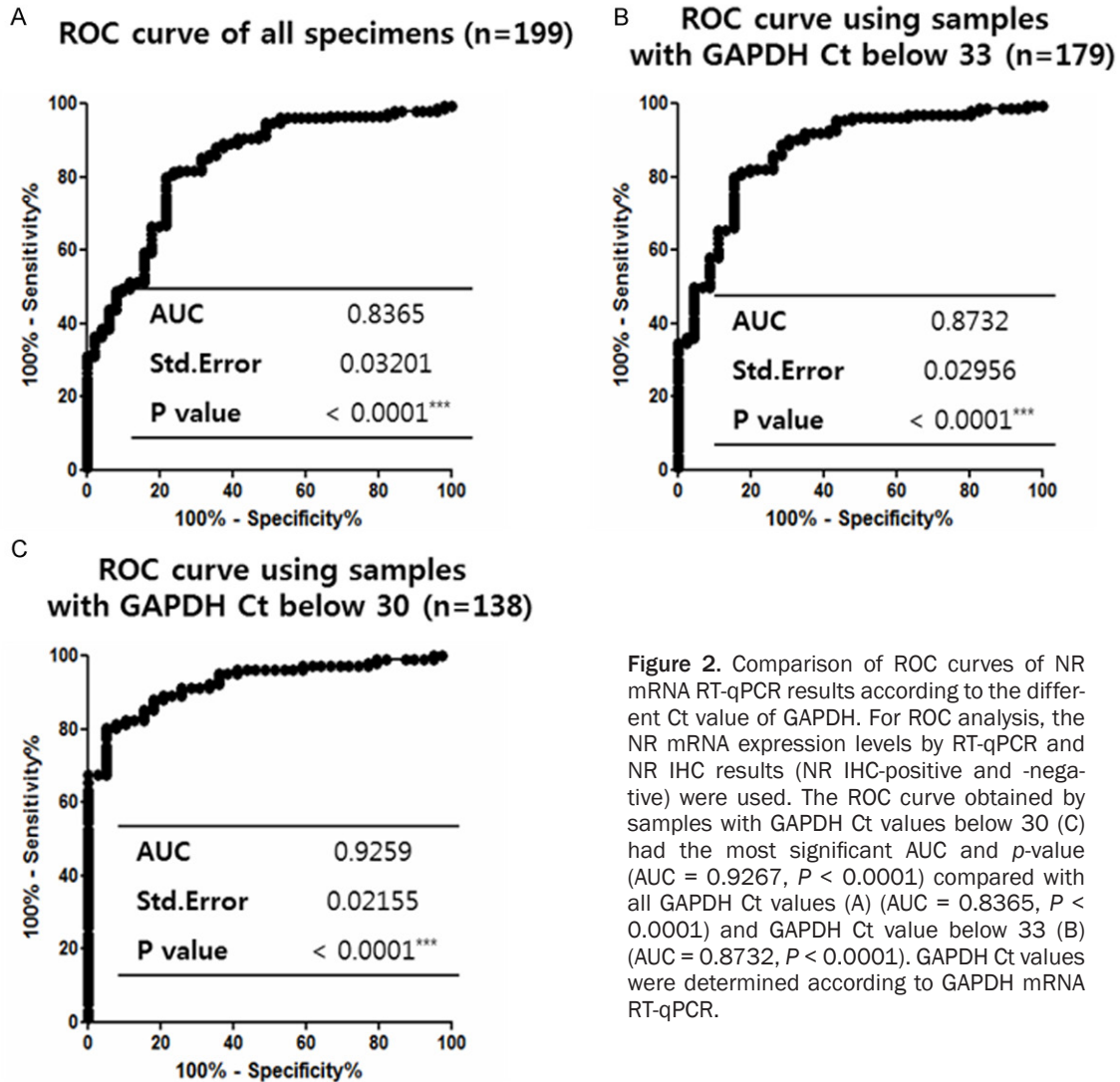
A

ROC curve of all specimens (n=199)

AUC	0.8365
Std.Error	0.03201
P value	< 0.0001***

B

ROC curve using samples with GAPDH Ct below 33 (n=179)

AUC	0.8732
Std.Error	0.02956
P value	< 0.0001***

C

ROC curve using samples with GAPDH Ct below 30 (n=138)

AUC	0.9259
Std.Error	0.02155
P value	< 0.0001***

Figure 2. Comparison of ROC curves of NR mRNA RT-qPCR results according to the different Ct value of GAPDH. For ROC analysis, the NR mRNA expression levels by RT-qPCR and NR IHC results (NR IHC-positive and -negative) were used. The ROC curve obtained by samples with GAPDH Ct values below 30 (C) had the most significant AUC and p-value (AUC = 0.9267, P < 0.0001) compared with all GAPDH Ct values (A) (AUC = 0.8365, P < 0.0001) and GAPDH Ct value below 33 (B) (AUC = 0.8732, P < 0.0001). GAPDH Ct values were determined according to GAPDH mRNA RT-qPCR.

Table 2. Clinical cut-off values of NR mRNA RT-qPCR obtained by ROC curve analysis with GAPDH Ct value below 30

Relative NR mRNA expression level	Sensitivity %	95% CI	Specificity %	95% CI	Likelihood ratio
> 96.4	81.2	72.2% to 88.3%	92.3	79.1% to 98.4%	10.6
> 98.4	80.2	71.1% to 87.5%	92.3	79.1% to 98.4%	10.4
> 103.3	80.2	71.1% to 87.5%	94.9	82.7% to 99.4%	15.6
> 107.3	79.2	70.0% to 86.6%	94.9	82.7% to 99.4%	15.5

Abbreviations: ROC, Receiver operating characteristic; CI, Confidence interval.

bated at 65°C for 5 min, and then quickly chilled on ice. A mixture of 4 μL of 5 × first-strand buffer, 2 μL of 0.1 M dithiothreitol (DTT), and 1 μL of M-MLV reverse transcriptase (RT) was added to the PCR tube. cDNA synthesis reaction was performed at 25°C for 10 min, 37°C for 50 min, and 70°C for 15 min.

RT-qPCR assay for the nuclear receptor detection

The relative mRNA expression levels of the nuclear receptors (NRs), ER and PR, were measured by a RT-qPCR employing TaqMan probes using a CFX-96 real-time PCR system (Bio-Rad,
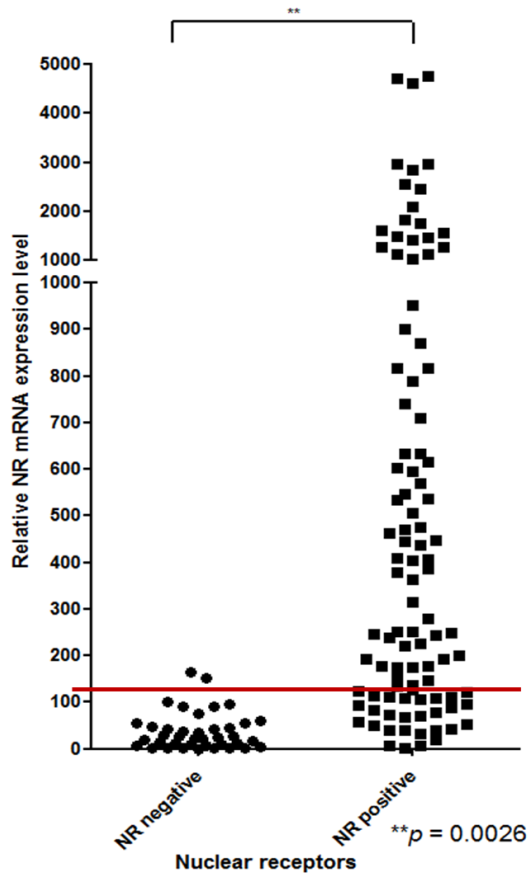
Figure 3. Evaluation of NR mRNA RT-qPCR according to the NR IHC score. FFPE breast cancer tissue samples were grouped by NR IHC results. The p-values for NR IHC-negative and -positive samples were calculated using Student's t-test ($P = 0.0026$).

Hercules, CA, USA), which was used for thermocycling and fluorescence detection. The RT-qPCR TaqMan assay was carried out with the 'BrightGen HR RT-qDX assay kit' (Syantra, Calgary, Canada) according to the manufacturer's protocols. Real-time PCR amplification for HER2 mRNA was performed using a total volume of 20 μL which was composed of 10 μL of 2 × Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 5 μL of primer and TaqMan probe mixture, 2 μL of template cDNA, and D.W. for each sample. Positive and negative controls were included throughout the procedure. No-template controls with sterile D.W. instead of template DNA were incorporated into each run under the following conditions: 95°C for 3 min, followed first by 10 cycles of 15 sec at 95°C and 30 sec at 60°C, then 40 cycles of 15 sec at 95°C and 30 sec at 55°C. The mRNA expression level was quantified by determining

the cycle threshold (C_T), which is the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence. To avoid false negatives due to degradation of mRNA, a GAPDH reference gene was used as a control. Target gene mRNA expression levels relative to GAPDH were automatically calculated using the comparative Ct method by CFX Manager Software v1.6 (Bio-Rad) or GenexSoftware (Bio-Rad), and the cut-off values for distinguishing between positive and negative results was the relative NR mRNA expression level of 103.3.

Statistical analysis

For statistical data analysis, 'GraphPad PRISM 5' software (GraphPad, La Jolla, CA, USA) was used. In order to determine statistical significance of the data, student's t-test and one-way ANOVA were carried out for the two-group comparison and multiple-group comparison, respectively. Receiver operating characteristic (ROC) curve analysis was applied to all the data of the NRs detection by IHC and RT-qPCR.

Results

Analytical sensitivity of the RT-qPCR for the NRs detection in breast cancer cell lines

In order to test whether the BrightGen HR RT-qDx used in this study was able to detect the NRs mRNA quantitatively, cDNA of breast cancer cell MCF7, which expresses high levels of ER and PR mRNA, was 10-fold diluted serially from 1×10^5 cells/mL to 1 cell/mL. The RT-qPCR for the NRs mRNA was performed three times. The mean Ct values of RT-qPCR using 10^5, 10^4, 10^3, 10^2, 10, and 1 cells/mL were 16.96, 20.09, 24.44, 28.26, and 34.89, respectively. The Ct value from the blank control reaction did not show any signal in any of the experiments (**Figure 1**).

Data were expressed as relative expression of the NRs to that in a calibrator cell line, MDA-MB-231. Expression levels of the NRs in MDA-MB-231 were set to 1X expression because MDA-MB-231 has low expression of ER and PR. Therefore, all the analyzed NR expression level was exhibited as fold increases toward the expression levels in the calibrator cell line. Two cell lines, MCF7 and THP-1, were employed as another control cell lines because they express higher level of the NRs: MCF7 expresses 93-fold higher and THP-1 expresses 52-fold

Table 3. Comparison of NR mRNA RT-qPCR results with NR IHC results

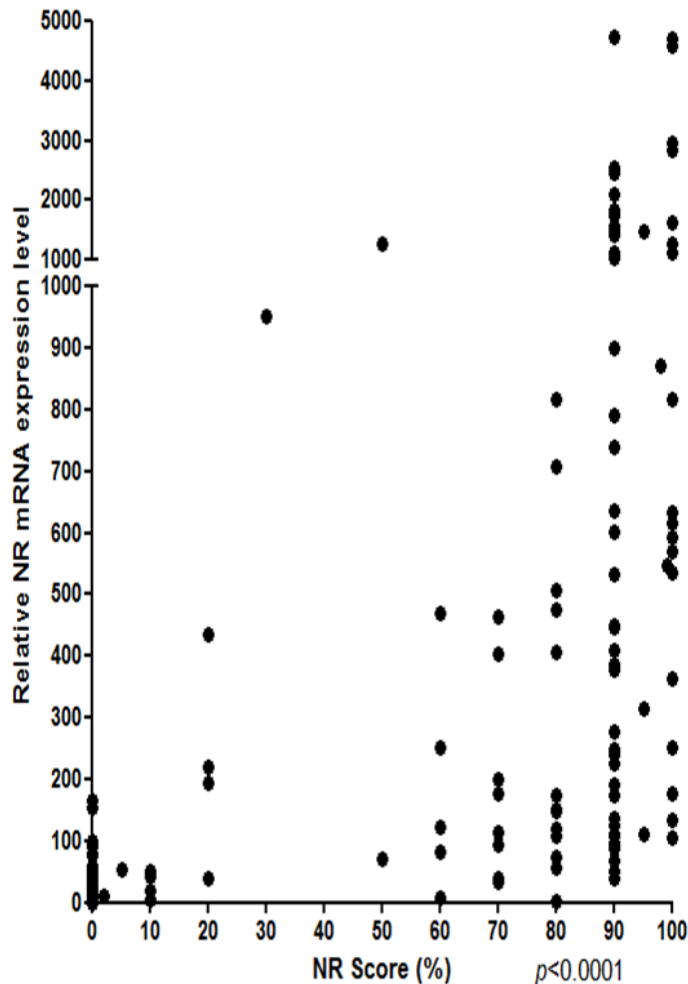| NR IHC status | NR mRNA RT-qPCR No. of samples (%) | | Total |
	Positive	Negative	
NR positive	83 (83.8)	16 (16.2)	99 (100)
NR negative	2 (5.1)	37 (94.9)	39 (100)
Total	93 (67.4)	47 (32.6)	138 (100)

Figure 4. Correlation coefficient analysis between the relative NR mRNA RT-qPCR and NR IHC results. The correlation between NR mRNA RT-qPCR and NR IHC results indicate a relationship (Pearson r = 0.4384, r^2 = 0.1922, P < 0.0001).

higher expression level of the NRs than that in MDA-MB-231 cell line, respectively (**Table 1**).

The range of relative expression level of the NRs, and diagnostic cut-off determination of the RT-qPCR assay

Relative expression level of the NRs in the FFPE samples detected negative with IHC for the

NRs ranged from 0.001 to 626 (average, 216.9), and IHC-positive FFPE samples ranged from 1.4 to 4705 (average, 1136.7).

ROC curve analyses and patients' IHC results were also used in order to determine the optimal cut-off value of the RT-qPCR assay for detection of the NRs in clinical samples. For the analysis, patients were divided into two groups: IHC-positive and IHC-negatives for the NRs. The IHC-positive group contained FFPE samples having IHC scores greater than 10% of ER and/or PR, whereas IHC-negative group contained IHC scores less than 10% of ER and PR results.

Area under the curve (AUC) obtained using the samples with any GAPDH Ct values (**Figure 2A**) was 0.8365, while AUC of the samples with GAPDH Ct values below 33 (**Figure 2B**) was 0.8732. AUC of the samples with GAPDH Ct values below 30 (**Figure 2C**) showed the highest score (AUC = 0.9259) indicating the highest sensitivity and specificity. The cut-off value of the RT-qPCR was determined by the likelihood ratio. The highest likelihood ratio was the relative NRs mRNA levels over 103.3 that had the highest sensitivity and specificity. Based on this result, samples having a relative NRs mRNA expression level greater than 103.3 by the RT-qPCR assay were considered positive in this study (**Table 2**).

Comparison between the RT-qPCR and IHC for detection of the NRs

The relative NRs mRNA levels in the FFPE samples were determined positive or negative using the clinical cut-off value obtained from the previous results (**Figure 3**). The samples were classified into two groups according to the NRs IHC results. The NRs-positive samples included over 10 samples having ER-positive. PR status was also used: IHC PR-positive status was over 10 cases. Of the NRs-positive IHC results, 83 samples were decided positive and 16 cases were decided negative by the RT-qPCR analyses. Among the samples IHC NR-negative, 37

were decided negative and two were decided positive by the RT-qPCR. The overall sensitivity and specificity of the the RT-qPCR for the NRs were 83.8% and 94.8%, respectively (**Table 3**). The *p*-values for the NRs IHC-negative and -positive samples were calculated using Student's *t*-test to be statically significant (*P* = 0.0026).

Correlation of the relative NRs mRNA expression levels with the NRs IHC scores

In order to verify the correlation between relative NRs mRNA expression levels and NRs IHC scores, correlation coefficient analysis was performed using the clinical samples having GAPDH Ct value below 30, depending on the results of the ROC curve analysis (**Figure 4**).

Samples having NR IHC score of below 10 showed 35, samples having NR IHC score of 10 to 50 showed 10, samples having NR IHC score of 60 showed 5, samples having NR IHC score of 70 showed 7, samples having NR IHC score of 80 showed 12, samples having NR IHC score of 90 showed 44, and samples having NR IHC score of 100 showed 19. Then, these NR IHC results were compared with the results obtained from the NR mRNA RT-qPCR analyses. The correlation coefficient analysis data revealed that there was a correlation between the results for the NRs expression by the RT-qPCR and standard IHC test (Pearson r = 0.4384, r^2 = 0.1922, *P* < 0.0001) (**Figure 4**).

Discussion

IHC is a standard method to detect expression of ER and PR in clinical samples for determining therapeutic application of Tamoxifen to breast cancer patients. IHC analysis also can be used for screening, diagnosis, and determination of cancer stage. Nevertheless, there are unchangeable weaknesses in IHC: time-consuming, inconsistent results between laboratories, and large dependency on antibodies used in the process [14, 15]. To complement the IHC analysis, we evaluated e a RT-qPCR assay as an alternative standard method for quantification of the NRs mRNA expression. RT-qPCR is considered a reliable method for quantification of mRNA expression recommended by the Clinical and Laboratory Standards Institute (CLSI, USA) guidelines. High sensitivity and specificity are usually achieved with the RT-

qPCR because primers and probes can be designed gene sequence-specific [16]. Therefore, we employed a RT-qPCR method for analyzing the NRs expression in the FFPE breast cancer tissue samples. Another strong point of the RT-qPCR might be involvement of an internal control: the NRs mRNA expression level was a relative fold increase toward the expression level of housekeeping gene, GAPDH.

The BrightGen HR RT-qDx assay kit used in this study is based on one-tube nested RT-qPCR. The one-tube nested RT-qPCR was known to approximately 100 times more sensitive than conventional qPCR [17]. Additionally, it has advantages of low risk of cross-contamination and less laborious procedure because all the sequential reactions are done in a single closed tube [17]. Actually, 83/99 (83.8%) samples were determined positive by BrightGen HR RT-qDx assay in our study (**Table 3**), which is higher than the 56/99 (56.6%) positivity determined by a conventional RT-PCR [18]. Thus, BrightGen HR RT-qDx kit, which based on one-tube nested RT-qPCR, was more sensitive than conventional qPCR for quantification of the NRs expression. Successful application of this RT-qPCR assay for clinical samples was evaluated using a total number of 138 FFPE tissue samples from breast cancer patients. Extraction of a high-quality and high-quantity of RNA from FFPE tissue samples is essential to obtain reliable results with RT-qPCR methods [19-21]. The quality and quantity of RNAs extracted from FFPE tissues were monitored by measuring Ct values of a housekeeping gene. Only when the RNA specimens met the criteria of quality and quantity, we used them for the analyses. In this study, RNAs having Ct values of GAPDH below 30 were used (**Table 2**). The sensitivity and specificity of the RT-qPCR for the NRs were 80.2% and 94.8%, respectively (**Figure 3** and **Table 3**). The discrepancy between the results obtained by the RT-qPCR and the IHC may be due to the low sensitivity of the RT-qPCR for the FFPE-derived RNA samples or false positivity of the IHC analyses. Further evaluation with larger number of FFPE breast cancer tissue specimens from various clinical fields is required to analyze the gap between the two assays.

In conclusion, this study shows that as long as good quality RNA is obtained from the stored

tumor tissues, the RT-qPCR method might be able to replace conventional IHC analysis for detection and quantification of ER and PR because the RT-qPCR method revealed high correlation with IHC for detection of the NRs in the clinical samples. Since many new biomarkers are being developed, we expect that the RT-qPCR can be applied to quantification of the biomarkers as a conventional analysis with high reproducibility and cost-efficiency

Acknowledgements

Disclosure of conflict of interest

None.

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