Development of RT-qPCR-based molecular diagnostic assays for therapeutic target selection of breast cancer patients

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Development of RT-qPCR-based molecular diagnostic assays for therapeutic target selection of breast cancer patients

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ABBREVIATIONS

ACK	:	ammonium-chloride-potassium
AUC	:	area under curve
cDNA	:	complementary DNA
CK19	:	cytokeratin 19
Ct	:	threshold cycle
CTC	:	circulating tumor cell
DCIS	:	ductal carcinoma in situ
DEPC	:	diethylpyrocarbonate
DTT	:	dithiothreitol
DMEM	:	Dulbeco's Modified Eagle's Medium
EtOH	:	ethanol
EpCAM	:	epithelial cell adhesion molecule
ER	:	estrogen receptor
FFPE	:	formalin fixed paraffin embedded
FISH	:	fluorescent *in situ* hybridization
GAPDH	:	glyceraldehyde 3-phophate dehydrogenase
HER2	:	human epidermal growth factor receptor 2
HR	:	hormone receptor
hTERT	:	human telomerase reverse transcriptase

IHC	:	immunohistochemistry
MMLV	:	Moloney murine leukemia virus
mRNA	:	messenger RNA
PBS	:	phosphate-buffered saline
PR	:	progesterone receptor
ROC	:	receiver operator characteristic
RT-qPCR	:	reverse transcriptase quantitative polymerase chain reaction
WHO	:	World Health Organization

ABSTRACT

Development of RT-qPCR-based molecular diagnostic assays for therapeutic target selection of breast cancer patients

Breast cancer is a significant cause of death in women. Human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) are important markers of targeted breast cancer treatment. Currently, the combination of fluorescence *in situ* hybridization (FISH) studies and immunohistochemical (IHC) staining are basic tests used to identify HER2, ER, and PR. If HER2 was found positive the test, Herceptin would be adopted for HER2-targeted treatment of breast cancer. If ER or PR is positive, estrogen inhibitors such as Tamoxifen will be administrated for ER-, PR- targeted cancer therapy. However, the procedures of IHC and FISH are invasive and involve difficulties in continuous observation of the patients' prognoses. In addition, because neither IHC nor FISH are standardized, test results can be varied depending upon the methods adopted. Therefore, in this study a molecular diagnostic

method based on RT-qPCR was developed to determine the expression of HER2, ER, and PR in more standardized manner. The results from this study showed that the HER2 RT-qPCR test developed in this study using patient's tissues exhibited 93.0% sensitivity and 89.9% specificity. On the other hand, the HR RT-qPCR test which can be used to test ER and PR using patient's tissues exhibited 80.2% sensitivity and 94.8% specificity. Subsequently, HER2 RT-qPCR was also conducted with blood specimens to examine whether the assay can detect HER2 mRNA expression in blood of breast cancer patients. The results showed that, over-expression of HER2 mRNA was not identified in blood of normal individuals, but identified in 20.1% of breast cancer patients' blood. In addition, HER2 mRNA was detected simultaneously with any one of EpCAM, CK19, Ki67, or hTERT, which are known circulating tumor cell markers. When the HER2 RT-qPCR results in the blood were compared with the histological HER2 results inconsistence were found, indicating breast cancer cells in the state of solid tumor may change when they are in the circulating blood. Therefore, the administration of Herceptin can be considered for patients who have an over-expressed HER2 mRNA in blood. In summary, the standardized

RT-qPCR based HER2 and HR tests would be helpful for treatment of breast cancer patients.

Key Words: human epidermal growth factor receptor 2 (HER2), hormone receptor (HR), RT-qPCR, Herceptin, Tamoxifen, target therapy, circulating tumor cell

PART 1.

Development of RT-qPCR-based molecular assays for target therapy of breast cancer patients

I. INTRODUCTION

Breast cancer remains the most common and second deadliest cancer in women [1]. The World Health Organization (WHO) estimates that worldwide, more than 508,000 women died in 2011 due to breast cancer. In the Republic of Korea, about 13,460 new cases of breast cancer were estimated for 2010.

Breast cancer is often considered a systemic disease because early tumor cell dissemination may even occur in patients with small tumors. The outcome of breast cancer largely depends on the development of metastases during the course of the disease.

Human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) are the biomarkers for breast cancer to be recommended for routine clinical use. HER2, ER, and PR exhibit both prognostic and predictive values.

HER2 is a member of the ErbB-like oncogene family, which consists of four closely related family members. Over-expression of *HER2* occurs in about 20~30% of breast cancer patients [2-6]. Genomic alterations of proto-oncogene *HER2* are associated with poor prognosis and a more aggressive tumor phenotype. A HER2 positive status indicates a poor prognosis and shorter overall survival time [7]. Patients with *HER2* amplification or over-

expression are eligible for treatment with Herceptin (Roche, Penzberg, Upper Bavaria, Germany) which is a humanized monoclonal antibody directed against the extracellular domain of the HER2. Herceptin is currently the standard first-line treatment of metastatic breast cancer, and it is also indicated in adjuvant settings, showing promising activity in neo-adjuvant treatment when combined with other chemotherapy [3,8,9]. The HER2 status stands alone in determining which patients are likely to respond to Herceptin.

In clinical studies of advanced breast cancers, Herceptin induced anti-tumor responses as a single agent and was more effective when combined with other chemotherapy. Based on these results, Herceptin was approved by the Food and Drug Administration (FDA) in 1998 for treatment of advanced breast cancer. Herceptin prolongs survival time in patients with operable HER2 positive cancer in adjuvant settings [10].

Several studies compared HER2 status between primary tumors and matched metastatic site samples [11,12]. All of these studies suggested an acceptable level of concordance between the HER2 status of the primary and the metastatic site samples (concordance rate: 80–94%).

In addition to HER2, steroid hormone receptors (HR), which include ER and PR, play important prognostic and biological roles in breast cancer. ER remains the single most important prognostic and predictive factor determining treatment outcomes [13]. ER is a member of the nuclear hormone family of intracellular receptors, and it plays a critical role in same cancer growth. Approximately 70% of breast cancer cases are ER-positive [14-17]. PR also plays significant roles in breast cancer biology. *PR*, an estrogen-regulated gene, is often co-expressed with *ER*, independently predicts breast cancer outcomes [18], and has been implicated in regulating stem-like cancer cells [19].

Tamoxifen, Raloxifene, and Fulvestrant are selective estrogen receptor modulators (SERMs) and function as ER antagonists in the breast. [20]. Anastrozole and Letrozole are inhibitors of aromatase which is an enzyme for production of estrogen. These selective drugs are used in ER- and PR-positive patients.

For this reason, HER2, ER, and PR tests are important for targeted therapy in breast cancer patients. The determination of HER2, ER, and PR on primary tumor tissues is routinely

performed by immunohistochemistry (IHC) or fluorescence *in situ* hybridization analysis (FISH) [21].

For HER2, IHC is the choice of methods, with 0/1+ signifying HER2-negative status and 3+ signifying HER2-positive status. Tumors showing a 2+ score by IHC are considered undetermined and need to be tested by FISH to conform their HER2 status [21,22]. The detection of ER and PR by IHC is slightly weak as a prognostic marker, but it is essential for the application of endocrine therapy, such as Tamoxifen-based therapy.

However, the intensity of IHC depends on the enzyme activity of horseradish peroxidase (HRP). Therefore, the staining intensity is significantly influenced by reaction time, temperature, and HRP substrate concentrations. The intensity of IHC often differs between experimenters who are well trained or not. Furthermore, HER2 IHC 2+ results need to require another test called FISH assay because of the insufficient sensitivity of IHC results in HER2 test. Since FISH assay uses fluorescence dyes, the stained slide can be not permanently preserved, and not every hospitals have capabilities to perform FISH assay [23,24].

Therefore, to overcome disadvantages of IHC and FISH

assays, a molecular technique based on the quantitative evaluation of target gene expression at the mRNA level has been proposed. Particularly in this study, reverse transcription quantitative PCR (RT-qPCR) targeting HER2 and HR at the mRNA levels were developed. The assays were then evaluated using FFPE clinical specimens which have IHC and FISH histological test results.

II. **MATERIALS AND METHODS**

1. Cell lines and cell culture

Human breast carcinoma cell line SK-BR3, over-expressing HER2 and weak-expressing ER and PR, was obtained from the Korean Cell Line Bank (Seoul, Korea). Human breast carcinoma cell line MCF7, over-expressing ER and PR and weak-expressing HER2, and MDA-MB-231, weak-expressing HER2, ER and PR, were obtained from the Yonsei Cancer Center (Seoul, Korea). THP-1 human monocytic cell line, weak-expressing HER2 and over-expressing ER, was obtained from Yonsei Medical School (Seoul, Korea) (Table 1).

The SK-BR3, MCF7, and MDA-MB-231 cancer cells were maintained as mono-layer cultures in DMEM at 37℃ under 5% CO_2. THP-1 cells were cultured in RPMI 1640 supplemented with 2 mM glutamine. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco-BRL, Carlsbad, California, USA).

Table 1. Expression levels of target genes in cell lines used in this study

Cell line	HER2 status	ER/PR status	Origin of Cell line
SK-BR-3	Over-expressing	Weak-expressing	Breast cancer
MCF7	Weak-expressing	Over-expressing	Breast cancer
MDA-MB-231	Weak-expressing	Weak-expressing	Breast cancer
THP-1	Weak-expressing	Over-expressing	Monocyte

2. RNA preparation from cultured cell

The $1x10^6$ cultured cells were used for RNA preparation. Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions. In brief, 1 ml of TRIzol was added to $1x10^6$ cells and was incubated for 5 min at room temperature. After that, 200 μl of chloroform was added to the cells and was centrifuged at 12,000 g for 15 min at 4℃. Then, the aqueous phase was transferred to a fresh 1.5 ml tube and 550 μl of isopropyl alcohol was added to the tube, which was then incubated for 10 min at room temperature. After incubation, the samples were centrifuged at 12,000 g for 10 min at 4℃. Subsequently, the supernatant were removed and 1 ml of 75% EtOH (in DEPC water) was added to the tube, mixed by vortexing which was centrifuged at 7,500 g for 5 min at 4℃. The supernatant was removed and dried in the air for 5 min. 50 μl of DEPC water or RNase free water was added. The final RNA solution was kept at -70℃ before use.

3. Clinical FFPE tissues

A total of 199 formalin-fixed paraffin-embedded (FFPE) tissue samples obtained from patients diagnosed with breast cancer at Yonsei Severance Hospital between 2009 and 2011 were subjected for this study. With all patient tissues, IHC was already performed to verify expression of HER2, ER, and PR according to IHC kit manufacture's protocol (Dako Cytomation, Glostrup, Denmark) at Yonsei Severance Hospital (Seoul, Korea). With the FFPE tissue from each paraffin block, two pieces of 10 μm-thick sections was used for RNA extraction. All subjects were provided with a written informed consent and the study was approved by the Institutional Ethics Committee of Yonsei Severance Hospital (approval number 1-2010-0018).

4. Deparaffinization of FFPE tissue

In order to remove paraffin from FFPE tissue samples, two pieces of FFPE tissue sections were put in a 1.5 ml microcentrifuge tube and 1 ml of 100% xylene was added to the FFPE sections. After shaking and vortexing, the tube was heated for 5 min at 50℃ for the paraffin to be melted and centrifuged for 2 min at room temperature at 20,000 g to pellet the tissue. After centrifugation, xylene was removed, and 1 ml of 100% EtOH was added to mix the sample, which was centrifuged at 20,000 g for 2 min at room temperature. After that, EtOH discarded without disturbing the pellet. The ethanol washing was 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.

5. Total RNA extraction of FFPE tissue

MagNA Pure LC RNA Isolation Kit III (Tissue) (Roche, Penzberg, Upper Bavaria, Germany) was used according to the manufacturer's protocol for total RNA extraction. In brief, 140 μl of Tissue Homogenized Buffer (Roche, Penzberg, Upper Bavaria, Germany) and 16 μl of 10% SDS solution were added to the deparaffinized tissue, respectively. Then, the mixed samples were vortexed and incubated overnight at 55℃. After incubation, 220 μl of Tissue Lysis Buffer (Roche, Penzberg, Upper Bavaria, Germany) was added to the tissue lysate supernatant. Then, MagNA Pure LC 2.0 (Roche, Penzberg, Upper Bavaria, Germany) machine was used to RNA preparation.

6. cDNA synthesis and RT-qPCR

The cDNA was synthesized using 2 μg of total RNA, 0.25 μg of random hexamers (Invitrogen, Carlsbad, California, USA), and 200 U Murine Molony Leukemia Virus Reverse Transcriptase (MMLV-RT; Invitrogen, Carlsbad, California, USA) with incubation for 10 min at 25℃, followed by 50 min at 37℃, and 15 min at 70℃.

mRNA levels of HER2, HR (ER and PR), and GAPDH were measured by RT-qPCR employing TaqMan probes in an ABI 7500 fast system (ABI, Carlsbad, California, USA). The total volume of RT-qPCR was 20 μl, which included 25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 200 μM each dNTP, 1 U *Taq* polymerase (TAKARA, Shiga, Japan), 1 pmole each forward and reverse primers, 1 pmole TaqMan probe, and 2 μl of cDNA. The thermal cycling conditions of HER2 RT-qPCR was 10 min at 95℃, followed first by 10 cycles of 15 sec at 95℃ and 30 sec at 60℃, then 40 cycles of 15 sec at 95℃ and 30 sec at 55℃. The conditions of HR RT-qPCR were 10 min at 95℃, followed by 40 cycles of 15 sec at 95℃ and 30 sec at 55℃.

Relative gene expression was assessed using the comparative Ct method (ΔΔCt method). The amount of target, normalized to an internal housekeeping gene, *GAPDH*, and relative to a calibrator, is given by $2^{-\Delta\Delta Ct}$ which was the normalized according to the following equation :

$$\Delta\Delta Ct=[\Delta Ct_{(test)}=Ct_{(target\ test)}-Ct_{(ref.\ test)}] - [\Delta Ct_{(calibrator)} = Ct_{(target\ calibrator)}-Ct_{(ref.\ calibrator)}]$$

Then, data were calculated the expression ratio by $2^{-\Delta\Delta Ct}$, that is represent relative mRNA level. Data expressed as relative HER2 mRNA levels and HR mRNA refer to a calibrator sample, the MDA-MB-231 cell line, which was chosen to represent 1x expression of *HER2* and *HR* gene. All analyzed mRNA expressed as n-fold HER2 and HR mRNA were relative to the calibrator.

Table 2. Oligonucleotide primers and TaqMan probes used in this study to detect mRNA levels of HER2 and HR

Target mRNA	Name of primer and probe	Sequence of primer and probe (5'→3')
HER2	HER2-F-1	AAC CTG GAA CTC ACC TAC CTG CCC AC
	HER2-R-1	AAC TCA CCT ACC TGC CCA CCA AT
	HER2-F-2	CGA TGA GCA CGT AGC CCT GCA C
	HER2-R-2	CAC GTA GCC CTG CAC CTC CT
	HER2-F-3	AAG CAT ACG TGA TGG CTG GTG T
	HER2-R-3	TCT AAG AGG CAG CCA TAG GGC ATA
	HER2-P-1	FAM-CAG CCT GTC CTT CCT GCA GGA TAT C-BHQ1
	HER2-P-2	FAM-ATA TGT CTC CCG CCT TCT GGG CAT CT-BHQ1
	HER2-P-3	FAM-CAT CCA CGG TGC AGC TGG TGA CAC A-BHQ1
HR	ER-F-1	TGC CAA ATT GTG TTT GAT GGA T
	ER-R-1	CGA CAA AAC CGA GTC ACA TCA GT
	ER-F-2	GCC AAA TTG TGT TTG ATG GAT TAA
	ER-R-2	GAC AAA ACC GAG TCA CAT CAG TAA TAG
	ER-P-1	FAM-CGA CAA AAC CGA GTC ACA TCA GT-BHQ1
	ER-P-2	FAM-ATG CCC TTT TGC CGA TGC A-BHQ1
	PR-F-1	AGT CAG AGT TGT GAG AGC ACT GGA
	PR-R-1	CTG GCT TAG GGC TTG GCT TTC ATT
	PR-P-1	FAM-TGC TGT TGC TCT CCC ACA GCC AGT-BHQ1
GAPDH	GH-F	CCA TCT TCC AGG AGC GAG ATC C
	GH-R	ATG GTG GTG AAG ACG CCA GTG
	GH-P	FAM-TCC ACG ACG TAC TCA GCG CCA GCA-BHQ1

7. Statistical Analysis

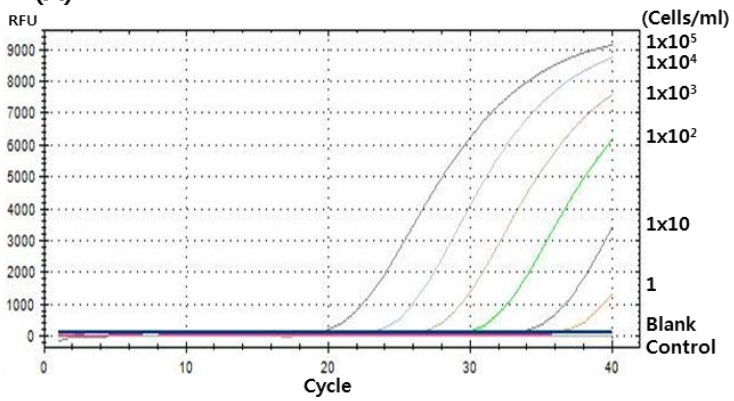
For statistical data analysis, the software PRISM 5 (GraphPad software, La Jolla, California, USA) was used. In order to determine the statistical significance of data, student's *t*-test and one-way ANOVA were carried out for the two-group comparison and multiple-group comparison, respectively. For correlation analyses between conventional IHC and RT-qPCR, the Spearman correlation coefficient was determined. ROC curve analysis was performed for all data.

III. **RESULTS**

1. The analytical sensitivity of HER2 RT-qPCR using reference cancer cell lines

In order to test whether the RT-qPCR designed in this study was able to detect HER2 mRNA quantitatively, cDNA of breast cancer cell SK-BR3, which expresses high levels of *HER2*, was serially 10-fold diluted from $1x10^5$ cells/ml to 1 cell/ml. RT-qPCR was performed three times, and the mean Ct values obtained from RT-qPCR using each serially diluted SK-BR3 cDNA are show in Figure 1. The mean Ct value of RT-qPCR using 10^5, 10^4, 10^3, 10^2, 10, and 1 cell(s)/ml was 19.45, 23.20, 26.49, 29.79, 33.35, and 36.08, respectively. The Ct value from the blank control reaction did not show any signal in all experiments (Figure 1).

(A)



(B)

Number of SK-BR3 (cells/ml)	Ct value
$1x10^5$	19.45
$1x10^4$	23.20
$1x10^3$	26.49
$1x10^2$	29.79
$1x10^1$	33.35
1	36.08
Blank control	*N.D.

Figure 1. The analytical sensitivity of HER2 RT-qPCR. (A) The analytical sensitivity of HER2 RT-qPCR was determined using serially diluted SK-BR3, a HER2 over-expressing cell line. (B) RT-qPCR was performed three times and the mean Ct value was calculated. (*N.D.; Not detected).

Subsequently, to test whether the HER2 RT-qPCR is useful to provide information on HER2 mRNA levels, various cell lines whose HER2 mRNA levels vary were subjected to HER2 RT-qPCR assay. Relative HER2 mRNA levels were measured by ABI 7500 (ABI, Carlsbad, California, USA). RT-qPCR data analysis was performed with ABI 7500 software c.2.04 (ABI, Carlsbad, California, USA), with the GAPDH gene as an internal housekeeping marker.

Data expressed as relative HER2 mRNA levels refer to a calibrator sample, the MDA-MB-231 cell line, which was chosen to represent 1x expression of *HER2* gene. All analyzed mRNA expressed as n-fold HER2 mRNA were relative to the calibrator.

According to the estimation mentioned above, MDA-MB-231, a HER2 weak-expressing cell line, was fixed to express a 1.0 relative HER2 mRNA level. As shown in Table 3 SK-BR3, a HER2 over-expressing cell line, showed about 57-fold higher mRNA level of HER2 than MDA-MB-231. Monocyte cell line THP-1, an HER2 weak-expressing cell line, showed about a 0.7 relative HER2 mRNA level.

Table 3. Relative HER2 mRNA levels detected by RT-qPCR in various cell lines

Cell line	HER2 status	Origin of cell	Relative HER2 mRNA level
SK-BR-3	Over-expressing	Breast cancer	56.99 ± 7.34
MCF7	Weak-expressing	Breast cancer	5.43 ± 2.25
MDA-MB-231	Weak-expressing	Breast cancer	1.0
THP-1	Weak-expressing	Monocyte	0.7 ± 0.2

2. HER2 RT-qPCR using clinical specimens

In order to determine whether HER2 RT-qPCR was useful to detect HER2 expression with clinical specimens, a total of 199 FFPE samples of breast cancer patients who had IHC scores were subjected to RT-qPCR of HER2 and GAPDH.

According to the HER2 RT-qPCR, the samples with IHC 0 score showed relative HER2 mRNA levels from 4.5 to 395 (Average 83.7). And the samples with IHC 1+ score showed relative HER2 mRNA levels from 0.5 to 401 (Average 86.6). The IHC score 2+/FISH negative showed relative HER2 mRNA level from 0.01 to 813 (Average 198.1). In HER2 positive groups, IHC 2+/FISH positive showed relative HER2 mRNA level from 13.3 to 2711 (Average 709.4) and IHC 3+ showed relative HER2 mRNA levels from 22.5 to 4850 (Average 1936.7).

3. Determination of the clinical cut-off value of HER2 RT-qPCR using receiver operating characteristic (ROC) curve analysis

It is very difficult to extract a high quality of RNA from clinical specimens such as FFPE tissues. In particular, when the relative mRNA levels need to be determined based on the reference housekeeping genes, one should be very careful. Thus, determining RNA quality is very important when working with RNA derived from FFPE specimens. High-quality RNA shows accurate results, whereas low-quality RNA shows false-positive or false-negative results [25-27]. Therefore, RNA quality obtained from 199 FFPE tissues was assessed based on the tissues' GAPDH expression level. The GAPDH expression levels of all the samples tested varied greatly.

In order to determine the optimal cut-off value for clinical use, ROC analysis was performed. For ROC analysis, the mRNA levels of HER2 using RT-qPCR and patient information about IHC were used.

For the analysis, a total of 199 patient's tissue samples were divided into two groups: HER2 IHC positives and negatives. In the HER2 IHC-positive group, IHC scores greater than 2+ and FISH positives were included. In the HER2 IHC-negative group,

IHC scores of 0 and 1+ were included. IHC 2+ and FISH-negatives were excluded from the assay because of the controversial nature of the clinical samples. The ROC curve analysis reveals the sensitivity and specificity by the area under curve (AUC) value. If the AUC value is closer to 1.0, the sensitivity and specificity are closer to 100%.

Since the Ct values of GAPDH RT-qPCR may represent the quality of the FFPE clinical samples, the ROC analyses were performed with three sets of clinical samples with different GAPDH Ct values.

GAPDH Ct values were determined by results of GAPDH RT-qPCR. GAPDH RT-qPCR were performed by using cDNA of breast cancer cell line that was serially 10-fold diluted from $1x10^5$ cells/ml to 1 cell/ml. RT-qPCR was performed three times, and the mean Ct values obtained from GAPDH RT-qPCR. The mean Ct value of GAPDH RT-qPCR using 10^5, 10^4, 10^3, 10^2, 10, and 1 cell(s)/ml was 19.00, 22.50, 26.00, 29.78, 33.32, and 36.50, respectively.

According to the results, FFPE samples were categorized by results of its GAPDH results. The criteria for ROC analysis were determined by GAPDH Ct below the 19, 23, 26, 30, 33, and 36.

Among 199 FFPE samples used in this study, the GAPDH Ct value below 36 included 196 FFPE samples, the GAPDH Ct value below 33 included 179 samples and the GAPDH Ct value below 30 included 153 samples. However, since the GAPDH Ct value below 26 included just 35 samples, this criteria was useless for the analysis. Therefore, GAPDH Ct values were divided into three groups for ROC curve analysis as shown in Figure 2. (A) all the samples with any GAPDH Ct values, (B) samples with GAPDH Ct values below 33 only, and (C) samples with GAPDH Ct values below 30 only.

As shown in Figure 2, the AUC value obtained using samples with GAPDH Ct values below 30 was the highest, indicating the highest sensitivity and specificity (AUC=0.9466). The samples with Ct values below 33 (Figure 2B) resulted in an AUC value of 0.9391, and the samples with any GAPDH Ct values (Figure 2A) resulted in an AUC value of 0.9225.

Subsequently, according to the ROC analysis, the cut-off value of HER2 RT-qPCR was determined by Likelihood ratio (Table 4). The highest Likelihood ratio was the relative HER2 mRNA levels over 105.5 folds, indicating highest sensitivity and specificity. Based on this result, relative HER2 mRNA levels

determined by HER2 RT-qPCR greater than 105.5 detected by HER2 RT-qPCR were determined a positive in this study.

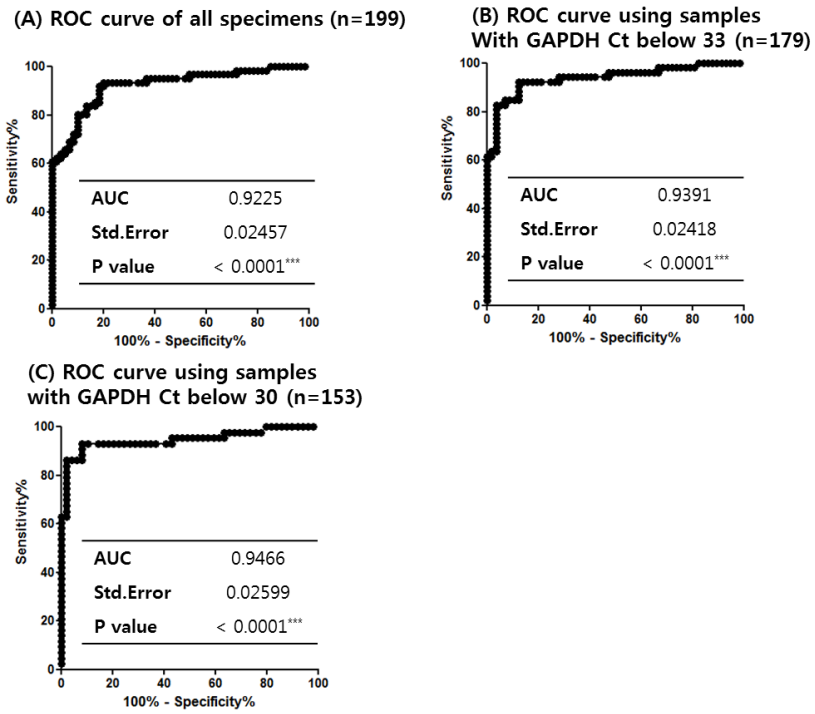


Figure 2. Comparison of ROC curves derived from HER2 RT-qPCR results using different GAPDH Ct values. For ROC analysis, the mRNA levels of HER2 using RT-qPCR and patients' information were used (HER2 IHC positive and negative). The ROC curve obtained by the samples with GAPDH Ct value below 30 (C) has shown the best significant AUC and *p* value (AUC=0.9466, $p<0.0001$) compared with all GAPDH Ct c value (A) (AUC=0.9225, $p<0.0001$) and GAPDH Ct value below 30 (B) (AUC=0.9391, $p<0.0001$). GAPDH Ct values are determined by GAPDH RT-qPCR results.

Table 4. Clinical cut-off values of HER2 RT-qPCR obtained by ROC curve analysis with GAPDH Ct value below 30

Relative HER2 mRNA level	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 73.90	93.02	80.94% to 98.54%	83.67	70.34% to 92.68%	6.51
> 78.10	93.02	80.94% to 98.54%	85.71	72.76% to 94.06%	9.12
> 105.5	93.02	80.94% to 98.54%	89.80	80.40% to 97.73%	11.4
> 135.1	90.7	77.86% to 97.41%	91.84	80.40% to 97.73%	11.11

4. Comparison of HER2 RT-qPCR results with HER2 IHC and FISH results

Subsequently, using the clinical cut-off value obtained from previous results, the relative mRNA levels of breast cancer FFPE samples were determined to be either positive or negative (Figure 3). The samples were classified into groups according to their HER2 IHC and FISH status.

As shown in Table 5, for HER2 positive (HER2 IHC 3+ and IHC 2+/FISH+), all samples were HER2 RT-qPCR positives, with the exception of three samples. For HER2 negative (HER2 IHC 0 and 1+), 44 samples were negative except 5 samples. Therefore, the overall sensitivity and specificity of the HER2 RT-qPCR were found to be 93.0% and 89.8%, respectively (Table 5). The *p* values between HER2 IHC negative and IHC positive were calculated by one-way ANOVA test and found to be statically significant ($p<0.0001$). As mentioned above, IHC 2+/FISH- negatives were excluded from the assay because of the controversial nature of the clinical samples.

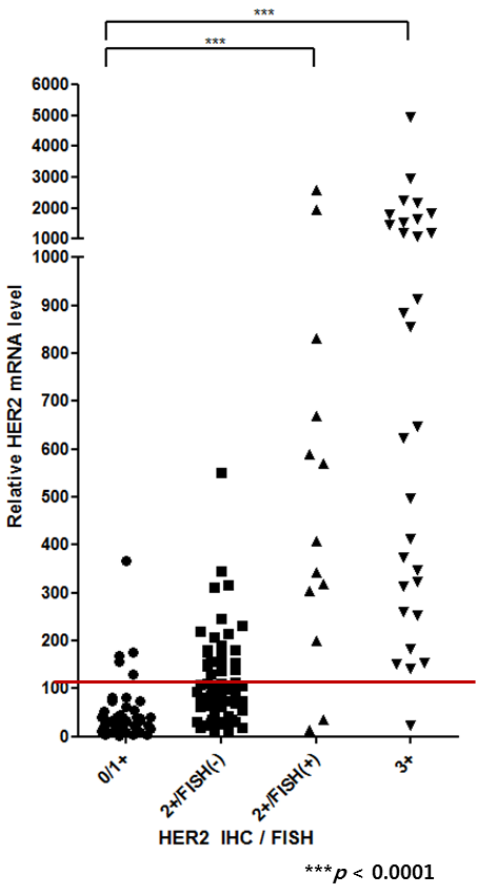


Figure 3. Evaluation of HER2 RT-qPCR according to the clinical cut-off value. Breast cancer FFPE tissues were grouped by HER2 IHC and FISH results. The *p* values between HER2 IHC negative and IHC positive were calculated using one-way ANOVA ($p<0.0001$).

Table 5. Comparison of HER2 RT-qPCR results with IHC and FISH results

IHC Score	HER2 RT-qPCR		Total
	Positive	Negative	
3+	29	1	30
2+/FISH(+)	11	2	13
2+/FISH(-)	28	33	61
0/1+	5	44	49
Total	73	80	153

5. Correlation of HER2 RT-qPCR results with HER2 IHC and FISH results

In order to verify the correlation between HER2 RT-qPCR and HER2 IHC/FISH, correlation coefficient analysis was performed using specimens of GAPDH below 30, depending on the results of the ROC curve analysis (Figure 4).

For the analysis of the correlation coefficient, the IHC test results were given as scores : a HER2 IHC score of 0 being 0, a HER2 IHC score of 1+ being 25, a HER2 IHC score 2+/FISH- being 50, a HER2 IHC score 2+ /FISH+ being 75, and a HER2 IHC score of 3+ being 100. Then, HER2 RT-qPCR results of FFPE tissues were compared with the IHC/FISH test results.

The correlation coefficient analysis confirmed a correlation between relative HER2 mRNA levels and clinical HER2 results (IHC and FISH) (Pearson r=0.5408, r^2=0.2936, p<0.0001).

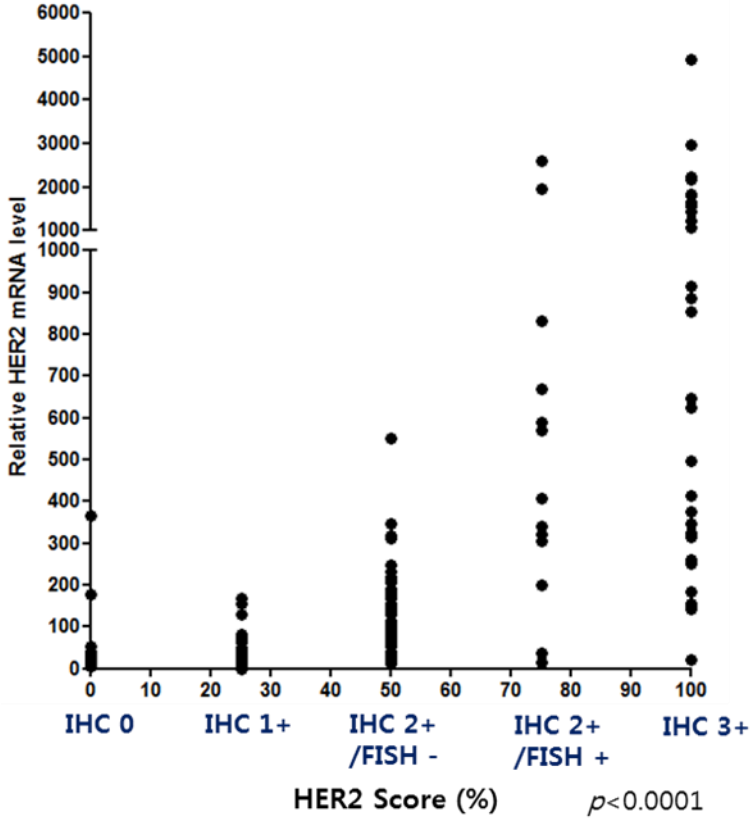


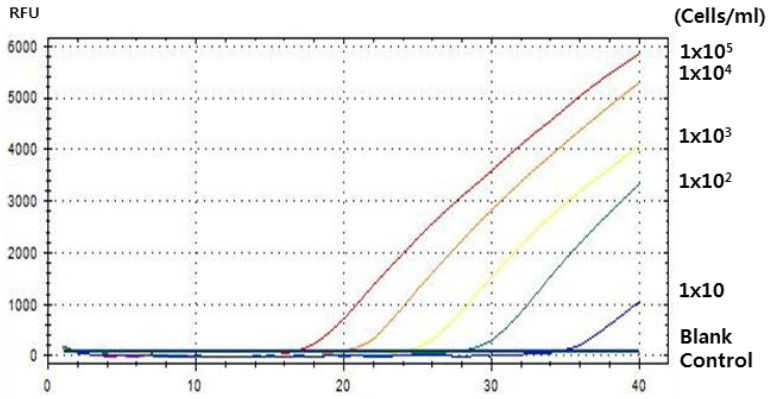
Figure 4. Correlation coefficient analysis between the HER2 RT-qPCR results and clinical HER2 results (IHC and FISH). The correlation coefficient analysis for HER2 RT-qPCR results and clinical HER2 results showed a good inter relations (Pearson r=0.5418, r^2=0.2936, p<0.0001).

6. The analytical sensitivity of HR RT-qPCR using reference cancer cell lines

Since ER and PR tend to express together in breast cancer tissues, patients with either ER or PR IHC positives get the same therapeutic agents such as tamoxifen. Nevertheless, ER and PR IHC tests need to be performed independently, which cost more time. Therefore, RT-qPCR was developed to target both ER and PR simultaneously in this study. RT-qPCR targeting both ER and PR is designated as HR RT-qPCR.

In order to test whether the RT-qPCR designed in this study is able to detect HR mRNA quantitatively, cDNA of breast cancer cell line MCF7, which expresses a high level of HR mRNA, was serially 10-fold diluted from $1x10^5$ cells/ml to 1 cell/ml. All RT-qPCR reactions were performed three times. The mean Ct value of RT-qPCR using 10^5, 10^4, 10^3, 10^2, and 10 cell(s)/ml was 16.96, 20.09, 24.44, 28.26, and 34.89, respectively (Figure 5). The Ct value from the blank control reaction did not show any signal in all experiments.

(A)



(B)

Number of MCF7 (cells/ml)	Mean Ct value
MCF7 1x10^5	16.96
MCF7 1x10^4	20.09
MCF7 1x10^3	24.33
MCF7 1x10^2	28.26
MCF7 1x10	34.89
Blank control	N.D.

Figure 5. The analytical sensitivity of HR RT-qPCR.

(A) The sensitivity of HR RT-qPCR was determined using serially diluted MCF7, a HR high expressing cell line. (B) RT-qPCR was performed three times and the mean Ct value was calculated.

(N.D.: Not detected).

Data were expressed as relative HR mRNA levels refer to a calibrator sample, the MDA-MB-231 cell line that was chosen to represent 1x expression of the *HR* gene. All analyzed mRNA expressed as n-fold HR mRNA were relative to the calibrator.

According to the estimation mentioned above, MDA-MB-231, a HR weak-expressing cell line, was fixed to express a 1.0 relative HR mRNA level. As shown in Table 6, MCF7, a HR over-expressing cell line showed about 93-fold higher mRNA level of HR than MDA-MB-231. Monocyte cell line THP-1, a HR over-expressing cell line, showed about 52-fold higher relative HR mRNA level.

Table 6. Relative HR mRNA levels detected by RT-qPCR in various cell lines

Cell line	ER/PR status	Origin of cell	Relative HR mRNA level
SK-BR-3	Weak-expressing	Breast cancer	3.66 ± 1.34
MCF7	Over-expressing	Breast cancer	92.78 ± 7.42
MDA-MB-231	Weak-expressing	Breast cancer	1.0
THP-1	Over-expressing	Monocyte	52.16 ± 4.23

7. Determination of the clinical cut-off value of HR RT-qPCR using ROC curve analysis

HR RT-qPCR was performed on a total of 199 FFPE samples of breast cancer patients provided by Yonsei Severance Hospital. The HR IHC-negative samples had relative HR mRNA levels ranging from 0.001 to 626 (Average 216.9), and HR IHC-positive samples ranged from 1.4 to 4705 (Average 1136.7).

In addition to HER2 RT-qPCR analysis, ROC curve analysis also was used in order to determine the clinical cut-off value for HR RT-qPCR. For ROC analysis, the mRNA levels of HR using RT-qPCR and patients' information were used. For the analysis, patients were divided into two groups: HR IHC positives and negatives.

In the HR IHC-positive group, IHC scores greater than 10% of ER and/or PR results were included. On the other hand, in the HR IHC-negative group, IHC scores less than 10 % of ER and PR results were included.

As shown in Figure 6, the AUC obtained using samples with GAPDH Ct values below 30 was the highest, indicating the highest sensitivity and specificity (AUC=0.9259). The samples with GAPDH Ct values below 33 (Figure 6B) resulted in an AUC

of 0.8732, and the samples with any GAPDH Ct values (Figure 6A) resulted in an AUC of 0.8365.

As shown in Table 7, the cut-off value of HR RT-qPCR was determined by Likelihood ratio. The highest Likelihood ratio was the relative HR mRNA levels over 103.3 that had highest sensitivity and specificity. Based on this result, relative HR mRNA levels of HR RT-qPCR greater than 103.3 were determined as positives in this study.

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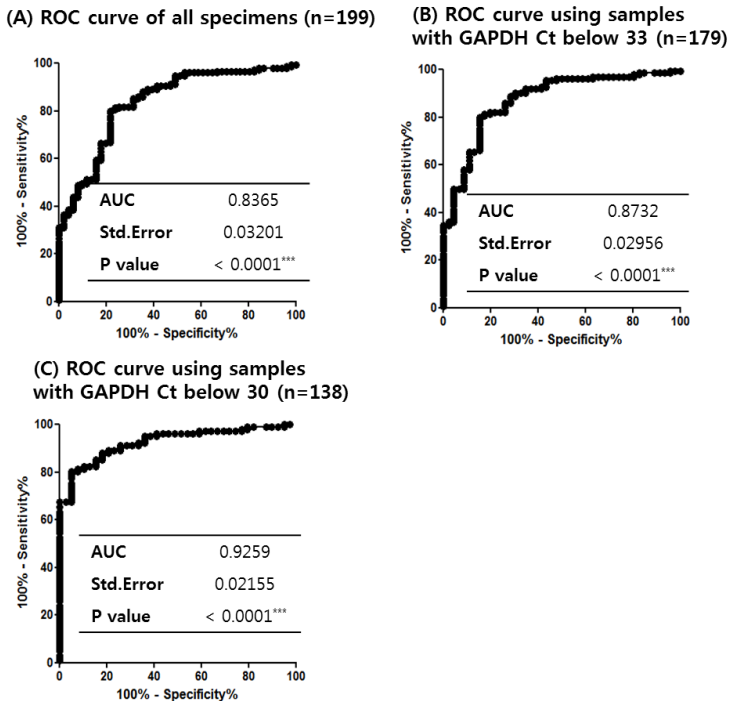


Figure 6. Comparison of ROC curves of HR RT-qPCR results using different GAPDH Ct values. For ROC analysis, the mRNA levels of HR using RT-qPCR and patients' information were used (HR IHC positive and negative). ROC curve obtained by samples with GAPDH Ct values below 30(C) had the most significant AUC and *p* value (AUC=0.9267, $p<0.0001$) compared with all GAPDH Ct value (A) (AUC=0.8365, $p<0.0001$) and GAPDH Ct value below 30 (B) (AUC=0.8732, $p<0.0001$). GAPDH Ct values are determined by GAPDH RT-qPCR results.

Table 7. Clinical cut-off values of HR RT-qPCR obtained by ROC curve analysis with GAPDH Ct value below 30

Relative HR mRNA level	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 96.35	81.19	72.19% to 88.28%	92.31	79.13% to 98.38%	10.55
> 98.40	80.2	71.09% to 87.46%	92.31	79.13% to 98.38%	10.43
> 103.3	80.2	71.09% to 87.46%	94.87	82.68% to 99.37%	15.64
> 107.3	79.21	69.99% to 86.64%	94.87	82.68% to 99.37%	15.45

8. Comparison of HR RT-qPCR results with IHC results

Subsequently, using the clinical cut-off value obtained from previous results, the relative mRNA levels of breast cancer FFPE samples were determined to be either positive or negative (Figure 7). The samples were classified into two groups according to their HR IHC status.

Breast cancer FFPE samples were analyzed by their HR IHC status using HR RT-qPCR. HR-positive samples in IHC including over 10 in ER IHC status. The PR status was also used, just like the ER status, and an IHC PR status over 10 was determined as being HR positive.

Of the HR-positive samples in IHC status, 83 samples were positive and 16 were negative for HR RT-qPCR results. For the HR-negative samples in IHC status, 37 were negative and two were positive. Therefore, the overall sensitivity and specificity of the HR RT-qPCR were found to be 80.2% and 94.8%, respectively (Table 8). The p values for HR IHC-negative and IHC-positive samples were calculated using Student's t-test and found to be statically significant (p=0.0026).

HR RT-qPCR had a higher specificity than HER2 RT-qPCR,

however, the sensitivity appeared lower than that of HER2 RT-qPCR.

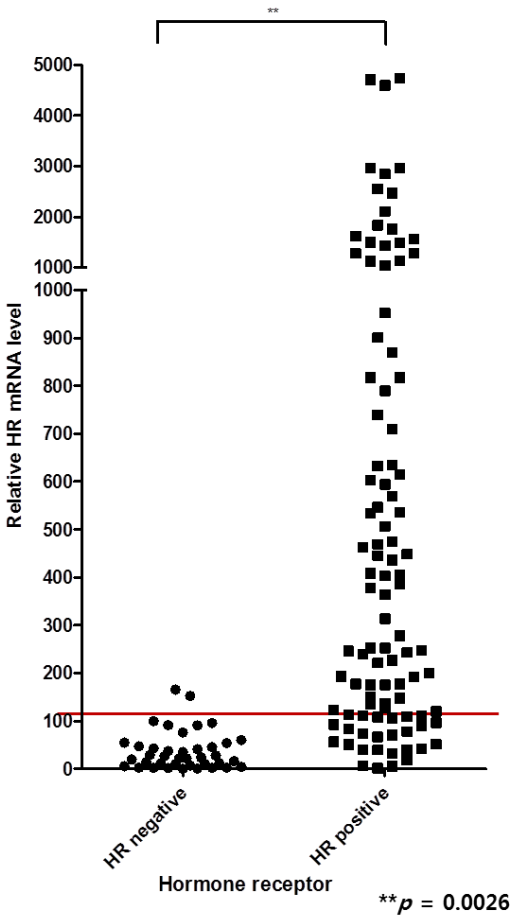


Figure 7. Evaluation of HR RT-qPCR according to the clinical cut-off value. Breast cancer FFPE specimens were grouped by HR IHC results. The *p* values for HR IHC-negative and IHC-positive samples were calculated using Student's *t*-test (p=0.0026).

Table 8. Comparison of HR RT-qPCR results and IHC results

	HR RT-qPCR		Total
	positive	negative	
HR positive	83	16	99
HR negative	2	37	39
Total	93	47	138

9. Correlation of HR RT-qPCR results with HR IHC results

In addition to HER2 RT-qPCR analysis, ROC curve analysis was also used to confirm that HR RT-qPCR was correlated with clinical HR data. Correlation coefficient analysis was performed using specimens of GAPDH below 30, depending on the results of the ROC curve analysis.

HR RT-qPCR results of FFPE tissues were compared with the IHC test results. The correlation coefficient analysis confirmed a correlation between relative HR mRNA levels and HR IHC results (Pearson r=0.4384, r^2=0.1922, p<0.0001) (Figure 8).

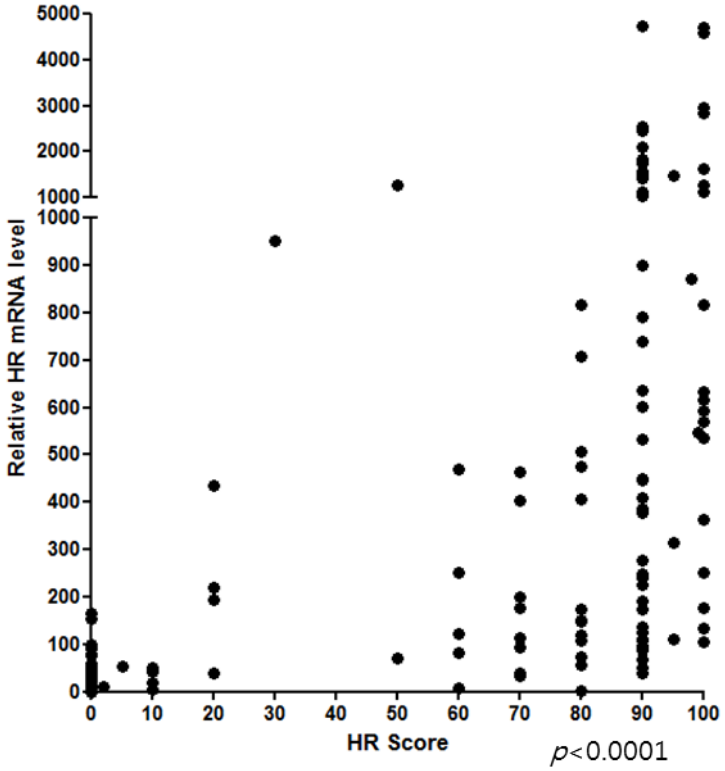


Figure 8. Correlation coefficient analysis between the relative HR RT-qPCR results and HR IHC results. The results of correlation coefficient analysis between HR RT-qPCR and clinical HR results showed a correlation (Pearson r=0.4384, r^2=0.1922, p<0.0001).

IV. DISCUSSION

IHC and FISH have been used as “gold standard” tests for target therapy that determines HER2 status for Herceptin administration and HR status for Tamoxifen administration in breast cancer patients. In particular, IHC has been most widely used, since it can be also used for screening, diagnosis, and the stage determination of cancer. However, IHC’s proficiency needs to be improved, as it is known that each laboratory does not provide the same technical accuracy and reproducibility of the results [21].

In comparison with IHC, FISH is known to yield good concordance and is more specific and sensitive. However, the FISH procedure is more complicated than IHC; furthermore, FISH has a weak point as a diagnostic test in that it cannot be a permanent stored since it uses fluorescent molecules. In addition, every hospital does not have capabilities to perform FISH assay [23,24].

For the reasons mentioned above, the aim of this study was set to develop new RT-qPCR-based molecular assays that can be used as companion tests for IHC and FISH used for HER2 and HR. RT-qPCR is now considered the gold standard for mRNA

quantitative evaluation, and its application to HER2 and HR status evaluation could contribute to method standardization and reduce reports of variability. RT-qPCR is a true quantitative technique, it is assessable according to Clinical and Laboratory Standards Institute guidelines, and it is highly sensitive and specific because primers and probes are sequence specific [28]. Therefore, RT-qPCR was applied to FFPE breast cancer specimens. The assays developed in this study measure the relative mRNA level of target genes in a reference to the housekeeping gene's expression level. Therefore, obtaining the minimum Ct value of the *GAPDH* gene expression level from specimens influenced the sensitivity and specificity of the assay.

For this study, FFPE tissue samples were used to evaluate the RT-qPCR-based tests developed in this study. It has been well documented that the extraction of a high-quality and a high-quantity of RNA from FFPE tissues require specific attention. The results from this study suggest that quality and quantity of the RNA extracted from FFPE tissues need to be monitored by Ct values obtained from the housekeeping gene targeting RT-qPCR, and the specimens only meet the certain criteria of the quality and the quantity of the RNA used for the assay. As shown in this

study, only the Ct values of GAPDH RT-qPCR below 30 should be used for the study (Figure 2 and Figure 6).

Using FFPE samples, RT-qPCR results were compared with the current gold standard methods. The main result of this study was that the determination of biomarkers was possible in clinical samples and showed significant correlation with the current diagnostic standard approaches. Based on the results, the overall clinical sensitivity of the HER2 RT-qPCR was 93.0% and the specificity was 89.8% (Figure 3 and Table 5). In particular, IHC 2+/FISH-negative but HER2 RT-qPCR-positive specimens will need further investigation.

In previous studies [29,30], it has been reported that even in patients whose IHC scores were 2+ but FISH results were negative, Herceptin had an effect on patients. In this study, there were 61 (39.9%) out of 153 patients whose IHC scores were 2+ and FISH results were negative. Currently, those 61 patients are not subjects for Herceptin treatment. However, of those 61 patients, the HER2 RT-qPCR testing performed in this study showed that 28 patients (45.9%) express the same high-level HER2 mRNA as the patients with FISH- positives. It would be very important to investigate whether those patients would

benefit from Herceptin target therapy.

HR RT-qPCR, targeting both ER and PR was also designed in this study, and the clinical usefulness of the assay was evaluated with 138 FFPE specimens from breast cancer patients. There were 99 ER and/or PR-positives among 138 patients. The new HR RT-qPCR detected 83 as positives. Therefore, the sensitivity and specificity of HR RT-qPCR was found 80.2% and 94.8%, respectively (Figure 7 and Table 8). The discordance between the new RT-qPCR based assay and the IHC may be due to the low sensitivity of the RT-qPCR with FFPE-derived RNA samples or due to the false positives determined by IHC. Further studies with larger amounts of breast cancer tissue specimens from various clinical fields need to be performed.

In this study, a quantitative RT-qPCR method was developed in order to improve the disadvantages of IHC and FISH. The results show that both HER2 RT-qPCR and HR RT-qPCR have high sensitivity and specificity. In addition, it seems that IHC- or FISH-negative patients and RT-qPCR-positive patients can be treated with Herceptin or Tamoxifen. Therefore, breast cancer patients will benefit from the study since more patients can be treated with the therapy suggested in this study.

In conclusion, this study shows that the method enables a good quality of RNA from the stored tumor tissues is important. The achieved agreement between IHC and RT-qPCR, and the large dynamic range and reproducibility of the new method opens a new rout in the field of personalized medicine. These methods may improve and predictive value of HR status. As many upcoming biomarkers from gene expression studies are measured on the mRNA level, this method is a major technical improvement for implementation of reproducible and cost-efficient testing of such biomarkers in clinical routine and in research studies using archived FFPE specimens in molecular pathology diagnostic testing.

PART 2.

Evaluation of HER2 RT-qPCR as a blood test for breast cancer target therapy

I. INTRODUCTION

The majority of breast cancer deaths are the result of recurrent metastasis. Metastasis occurs as a result of a series of steps involving multiple host-tumor interactions. Primary tumor cells may eventually detach and intravasate into the lymphatic and/or blood systems before developing into secondary disease. In breast cancer, when tumor cells enter the lymphatic system, they travel to the sentinel nodes in the axilla and intercostal spaces before entering the bloodstream and subsequently progressing to other organs. In addition, tumor cells can disseminate directly through the blood to distant organs. The detection and characterization of such cells would be an advantage in the identification of patients who are at risk of disease recurrence and the subsequent tailoring of individualized treatments [33-35].

Cancer cells which become detached from the primary tumors and enter into the systemic circulation are called circulating tumor cells (CTCs) they have physiological and biological similarities to the primary tumors [36,37]. The existence of CTCs is very important for the treatment and prognosis of breast cancer [38,39]. The presence of CTCs in the

blood of early-stage breast cancer patients increases the chance of recurrence and the five-year survival rate by 25% to 40% compared to patients with no CTCs [40-42].

Therefore, the detection of CTCs may have important prognostic and therapeutic treatment implications. There have been many studies conducted to detect the presence of CTCs using molecular methods [43-46]. Several markers, corresponding to molecules encoded by genes that are thought to be tissue specific and expressed on epithelial but not on hematopoietic cells, have been targeted to detect occult tumor cells in the peripheral blood of patients with breast cancer. Among these markers are epithelial adhesion molecule (EpCAM), cytokeratin 19 (CK19), antigen Ki67 and human telomerase reverse transcriptase (hTERT) [47-50].

EpCAM and CK19 are markers used to detect the specific epithelial antigens that are used to determine whether cancer cells are present in the blood of breast cancer patients. Antigen Ki67 is a nuclear protein that is associated with cellular proliferation. hTERT is catalytic subunit of the enzyme telomerase; it is associated with cancer cell immortalization. These markers, Ki67

and hTERT, are associated with cancer cell proliferation in the blood of cancer patients

HER2 is a member of the ErbB-like oncogene family [31]. HER2 testing is very important for the treatment of breast cancer. HER2 is over-expressed in 20% to 30% of breast cancer patients with a poor prognosis and more aggressive breast cancers. The five-year survival rate for breast cancer in such patients is less than in patients without the over-expression of HER2. Surgical therapy for breast cancer focuses on biopsy of the primary tumor in order to detect HER2 over-expression [7,32]. Therefore, it is necessary to develop a method to determine HER2 status of breast cancer cells using blood which can be obtained by rather noninvasive procedures than tissue specimens and can be used repeatedly.

The aim of this study was to evaluate the usefulness of HER2 RT-qPCR for detection of *HER2* expressing CTCs in the blood. In this study, over-expression of HER2 mRNA at the mRNA levels using the blood of breast cancer patients was tested by HER2 RT-qPCR.

Furthermore, correlations between these CTC markers and HER2 mRNA levels in the blood were determined. And in order

to the CTC related markers were associated with breast cancer stage, RT-qPCR results were grouped by breast cancer stage.

II. **MATERIALS AND METHODS**

1. Cell lines and cell culture

Human breast carcinoma cell line SK-BR3, over-expressing HER2, EpCAM, CK19, and Ki67, was obtained from the Korean Cell Line Bank (Seoul, Korea). Human breast carcinoma cell lines MCF7 and MDA-MB-231, weak-expressing HER2, were obtained from Yonsei Cancer Center (Seoul, Korea). MCF7 was over-expressing Ki67 and weak-expressing hTERT. MDA-MB-231 was over-expressing hTERT, but weak-expressing other markers (Table 9).

The SK-BR3, MCF7, and MDA-MB-231 were maintained as mono-layer cultures in DMEM at 37℃ under 5% CO_2. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco-BRL, Carlsbad, California, USA).

Table 9. Expression levels of CTC-related genes in each cell lines used in this study

Cell line	EpCAM	CK19	Ki67	hTERT
SK-BR3	+++	+++	+++	-
MCF7	++	++	+++	-
MDA-MB-231	+	+	+++	+++

2. Patients

A total of 188 breast cancer patients who had operations for breast cancer with ductal carcinoma in situ (DCIS) and stage I–III at the Yonsei Severance Hospital from 2011 to 2012 were included in the study. A total of 50 healthy donors who did not have a breast cancer were also included in this study.

All patients did not perform any neo-adjuvant chemotherapy and underwent routine IHC analysis for HER2 status in Yonsei Severance Hospital. Thirty patients had HER2-positive tumors, and the remaining 158 patients had HER2-negative breast cancer.

All patients provided with a written informed consent, and the study was approved by the Institutional Ethics Committee of Yonsei Severance Hospital (approval number 1-2010-0018). All healthy donors also provided with a written informed consent, and the study was approved by the Institutional Ethics Committee of Yonsei University at Wonju (approval number 2012-2).

3. Blood collection and RNA preparation

All blood samples were obtained at the middle of vein puncture after the first 5 ml of blood was discarded to avoid contamination of blood with epithelial cells during sample collection. The blood samples were lysed by ACK solution (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA). The 7.5 ml of blood sample was transferred into the 50 ml conical tube, and 42.5 ml of 1x ACK solution was added. After vortexing, the tube was incubated for 10 min at room temperature and was centrifuged for 10 min at 600 g at 4℃. After centrifugation, supernatant was removed in the tube and after 5 μl of RNase A was added the 1 ml of 1x PBS, incubated 5 min at room temperature. After incubation, samples were centrifuged for 2 min at 3,000 g at 4℃, the supernatant was removed, and 1 ml of TRIzol Reagent was added (Invitrogen, Carlsbad, California, USA). After that, 200 μl of chloroform was added to the cells and was centrifuged at the 12,000 g for 15 min at 4℃. Then, the aqueous phase was transferred to a fresh 1.5 ml tube and 550 μl of isopropyl alcohol was added to the tube, which was then incubated for 10 min at room temperature. After incubation, the samples were centrifuged at 12,000 g for 10 min at 4℃.

Subsequently, the supernatant were removed and 1 ml of 75% EtOH (in DEPC water) was added to the tube, mixed by vortexing, which was centrifuged at 7,500 g for 5 min at 4℃. The supernatant was removed and dried in the air for 5 min. 50 μl of DEPC water or RNase free water was added. The final RNA solution was kept at -70℃ before use.

4. cDNA synthesis and RT-qPCR

The cDNA was synthesized using 2 μg of total RNA, 0.25 μg of random hexamers (Invitrogen, Carlsbad, California, USA), and 200 U Murine Molony Leukemia Virus Reverse Transcriptase (MMLV-RT: Invitrogen, Carlsbad, California, USA) with incubation for 10 min at 25℃, followed by 50 min at 37℃, and 15 min at 70℃.

The mRNA levels of HER2, EpCAM, CK19, Ki67, hTERT, and GAPDH were measured by RT-qPCR based on the TaqMan probes in an ABI 7500 fast system (ABI, Carlsbad, California, USA). The total volume of RT-qPCR was performed in a total volume of 20 μl , which included 25 mM TAPS (pH 9.3 at 25℃), 50 mM KCl, 2 mM $MgCl_2$, 1mM 2-mercaptoethanol, 200 μM each dNTP, 1 U *Taq* polymerase (TAKARA, Shiga, Japan), 1 pmole Forward and Reverse primers, 1 pmole TaqMan probe, and 2 μl of cDNA. The thermal cycling conditions of HER2 RT-qPCR were 10 min at 95℃, followed by the first 10 cycles of 15 sec at 95℃ and 30 sec at 60℃, and second 40 cycles of 15 sec at 95℃ and 30 sec at 55℃. The conditions of RT-qPCR detection for the rest of markers were 10 min at 95℃, followed by 40

cycles of 15 sec at 95℃ and 30 sec at 55℃.

The mRNA levels of HER2 and CTC-related markers were calculated by ABI 7500 software c.2.04 (ABI, Carlsbad, California, USA), with the GAPDH. The data were expressed as standard internal housekeeping gene.

Relative gene expression was assessed using the comparative Ct method (ΔΔCt method). The amount of target, normalized to an internal housekeeping gene, *GAPDH* gene, and relative to a calibrator, is given by $2^{-\Delta\Delta Ct}$. which was the normalized according to the following equation :

$$\Delta\Delta Ct=[\Delta Ct_{(test)}=Ct_{(target\ test)}-Ct_{(ref.\ test)}] - [\Delta Ct_{(calibrator)} = Ct_{(target\ calibrator)}-Ct_{(ref.\ calibrator)}]$$

Then, data were calculated as the expression ratio by $2^{-\Delta\Delta Ct}$, that is represent relative mRNA level.

Relative mRNA levels of HER2, Ki67, and hTERT were referred to a calibrator, the normal blood samples, chosen to represent 1x expression of each gene. All analyzed tumors expressed as n-fold HER2, Ki67, and hTERT mRNA relative to the calibrator. Epithelial markers, EpCAM and CK19, were regarded as positive when the Ct value was under 39.

Table 10. Oligonucleotide primers and TaqMan probes designed in this study to detect the expression level of CTC markers

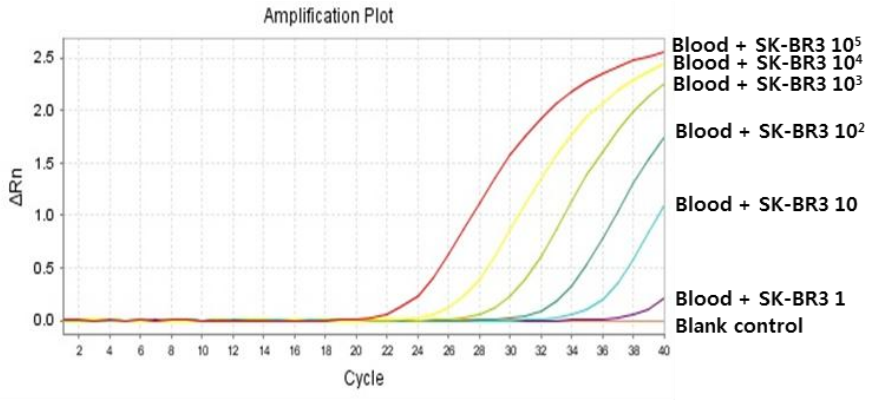
Target	Name of primer and probe	Sequence of primer and probe (5'→3')	Type of marker
EpCAM	Ep-F	GCC AGT GTA CTT CAG TTG GTG CAC	Epithelial Marker
	Ep-R	CAT TTC TGC CTT CAT CAC CAA ACA	
	Ep-P	FAM-TAC TGT CAT TTG CTC AAA GCT GGC TGC CA -BHQ1	
Cytokeratin 19	CK19-F	GAT GAG CAG GTC CGA GGT TA	
	CK19-R	TCT TCC AAG GCA GCT TTC AT	
	CK19-P	FAM-CTG CGG CGC ACC CTT CAG GGT CT-BHQ1	
Human telomerase reverse transcriptase	hTERT-F	TGA CGT CCA GAC TCC GCT TCA T	Cancer association Marker
	hTERT-R	ACG TTC TGG CTC CCA CGA CGT A	
	hTERT-P	FAM-GCT GCG GCC GAT TGT GAA CAT GGA -BHQ1	
Protein Ki67	Ki67-F	TAA TGA GAG TGA GGG AAT ACC TTT G	
	Ki67-R	AGG CAA GTT TTC ATC AAA TAG TTC A	
	Ki67-P	FAM-GGC GTG TGT CCT TTG GTG GGC A-BHQ1	

III. **RESULTS**

1. The analytical sensitivity of HER2 RT-qPCR in blood specimens

In order to find out whether HER2 RT-qPCR would detect HER2-expressing breast cancer cells in blood, the HER2-expressing cell line SK-BR3 was spiked with normal blood and used for HER2 RT-qPCR. From a total of $1x10^5$ cells/ml to 1 cell/ml, SK-BR3 cells were mixed with normal blood and the cDNA prepared from each sample was subjected to the assay. As shown in Figure 9, the Ct value of HER2 RT-qPCR with 1 cell level of SK-BR3 was lower than 40, which was the amplification cycle number of the assay.

(A)



(B)

Number of cell	Mean Ct value
Blood + SK-BR3 1x10^5	21.27
Blood + SK-BR3 1x10^4	24.28
Blood + SK-BR3 1x10^3	27.38
Blood + SK-BR3 1x10^2	30.78
Blood + SK-BR3 1x10	33.54
Blood + SK-BR3 1	37.55
Blank control	N.D.

Figure 9. The analytical sensitivity of HER2 RT-qPCR with SK-BR3 cells mixed with normal blood.

To determine the sensitivity of HER2 RT-qPCR in blood, normal blood mixed with SK-BR3 was used. The ten-fold serial diluted the number of SK-BR3 cell were spiked in normal blood.

(N.D. : Not detected)

The relative HER2 mRNA levels in healthy subjects were between 0.001 and 1.5. On the other hand, when a total of 188 blood specimens from breast cancer patients from Yonsei Severance Hospital were subjected to HER2 RT-qPCR, the relative HER2 mRNA levels ranged from 0.01 to 810.

For the relative mRNA levels measured by the HER2 RT-qPCR assay to be clinically informative, it was necessary to find out whether the HER2 RT-qPCR assay indeed detects HER2-expressing breast cancer cells in blood. Thus, in order to determine the usefulness of the assay, relative levels of HER2 mRNA higher than 10 were regarded as HER2-positive, whereas relative expression levels of HER2 mRNA lower than 10 were regarded as HER2 negative, and the characteristics of those samples with relative HER2 mRNA levels higher than 10 were investigated further. The *p* values for healthy donors and breast cancer patients were calculated using Student's *t*-test ($p < 0.0001$) (Figure 10).

In summary, out of 188 patients, 39 patients (20.7%) displayed an over-expression of HER2 mRNA, while none of the healthy blood donors over-expressed HER2 mRNA (Table 11).

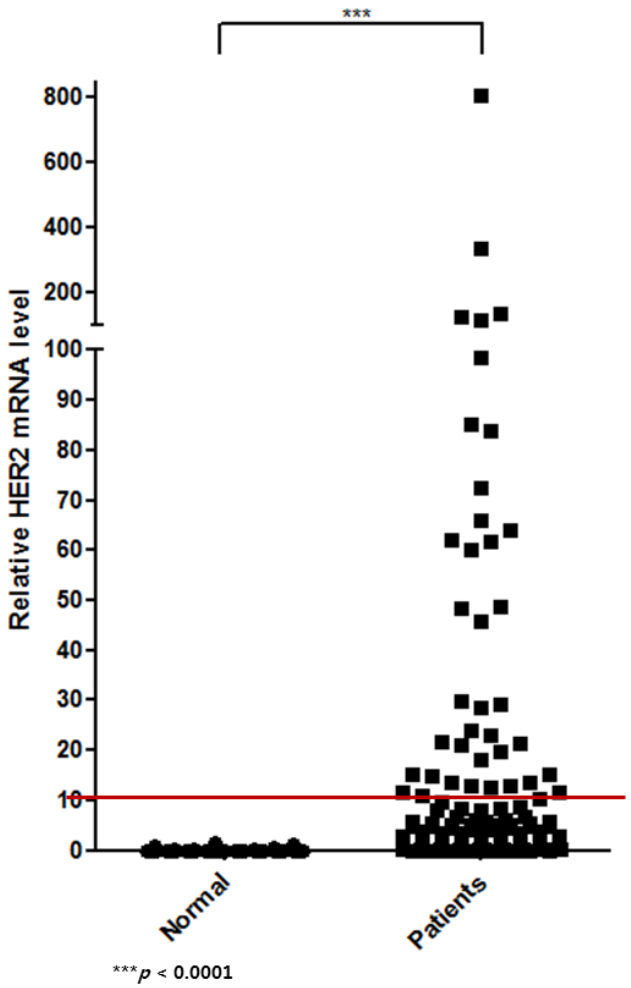


Figure 10. Comparison of relative HER2 mRNA levels in the blood of normal donors and breast cancer patients. The relative HER2 mRNA level of the healthy donor group was significantly lower than that of breast cancer patients. Thirty-nine breast cancer patients had relative HER2 mRNA levels greater than ten. The *p* values for healthy donors and breast cancer patients were calculated using Student's *t*-test ($p < 0.0001$).

Table 11. HER2 positivity of blood samples by HER2 RT-qPCR

n (%)	Healthy subject	Breast cancer patients
Blood HER2 (+)	0 (0)	39 (20.7)
Blood HER2 (–)	50 (100)	149 (79.3)
Total (n)	50 (100)	188 (100)

2. Development of RT-qPCR targeting biomarkers for CTC in the blood

If HER2 RT-qPCR detects the HER2-expressing breast cancer cells in patients' blood, the cells in the blood may have characteristics of CTC. In order to determine whether HER2-expressing cells in patients' blood have the CTC biomarkers such as EpCAM, CK19, Ki67, and/or hTERT, RT-qPCR assays that can detect such biomarkers were developed.

The breast cancer cell line SK-BR3 was used for the EpCAM and CK 19-based detection, the sensitivity of the Ki67 expression was observed mixed with MCF7 cells in normal blood, and the MDA-MB-231 human breast cancer cells were used for the hTERT-based detection.

As shown in Figure 11, the detection sensitivity for EpCAM suffices for even one tumor cell in the blood, whereas that for CK19, hTERT, and Ki67 can detect up to 10 cells of breast cancer cell lines.

The Ct values from the RT-qPCR amplification for EpCAM and CK19 mRNA in the blood of breast cancer patients were compared with those of normal blood. The relative HER2 mRNA levels of hTERT and Ki67 in the blood of the breast cancer

patients were compared with the normal blood group as well. In Figure 12, the Ct values were over 39 (the total cycle number being 40) or all the samples with EpCAM and CK19 in the normal blood excluding one sample with EpCAM, whereas the Ct values for all breast cancer patients were below 38. The percentage of EpCAM-positive patients was 45.2%, and 50.5% were CK19-positive. The expression of hTERT and Ki67 revealed that no healthy person displayed a relative hTERT mRNA level higher than 10; 20.7% of the breast cancer patients with hTERT and 13.8% of the breast cancer patients with Ki67 displayed an expression level higher than 10.

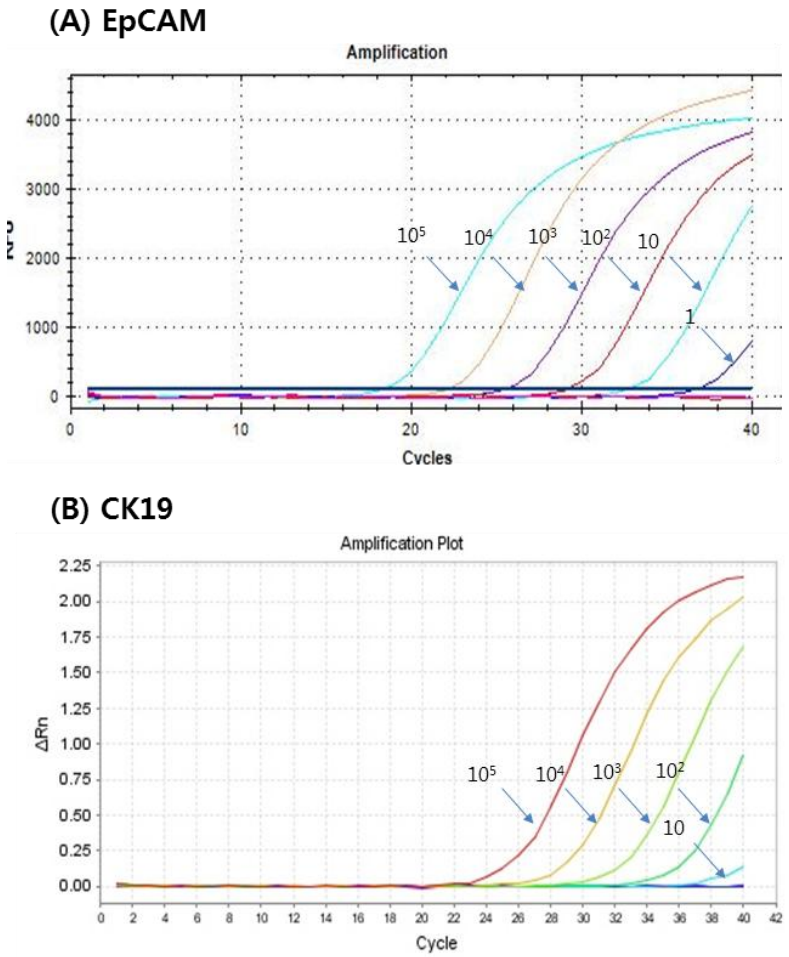


Figure 11. The analytical sensitivity of the CTC related markers in the blood. To determine the sensitivity of HER2 RT-qPCR in the blood, normal blood mixed with breast cancer cell lines was used. (A) EpCAM and (B) CK19 were expressed in blood mixed with SK-BR3.

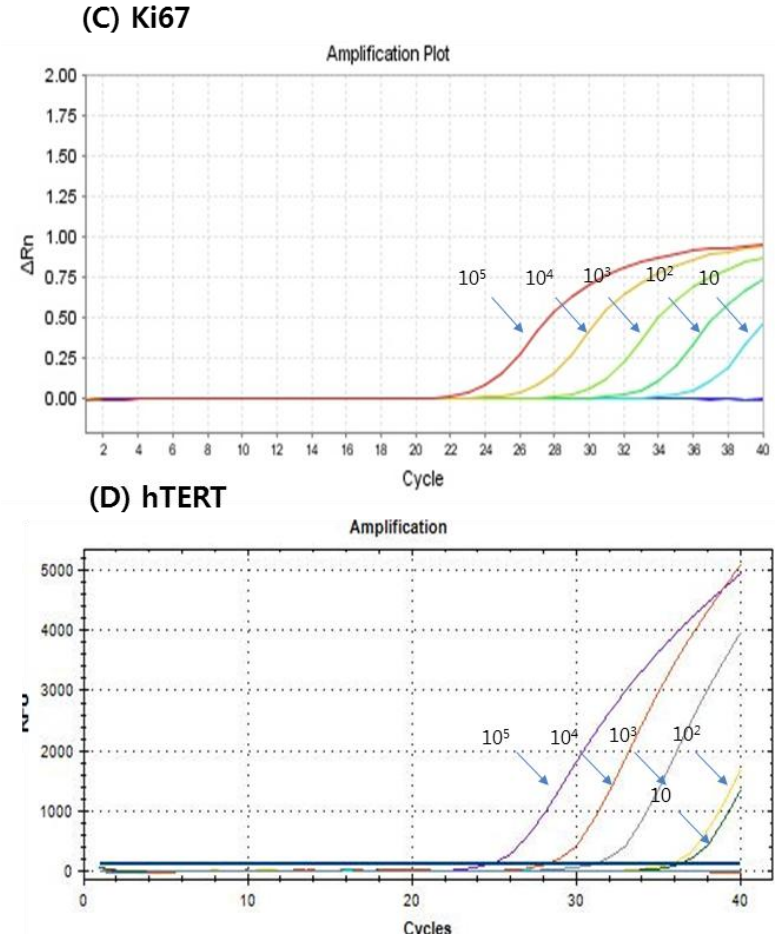


Figure 11. The analytical sensitivity of the CTC related markers in the blood (Continued). (C) Ki67 was expressed in blood mixed with MCF7. (D) hTERT was expressed in blood mixed with MDA-MB-231.

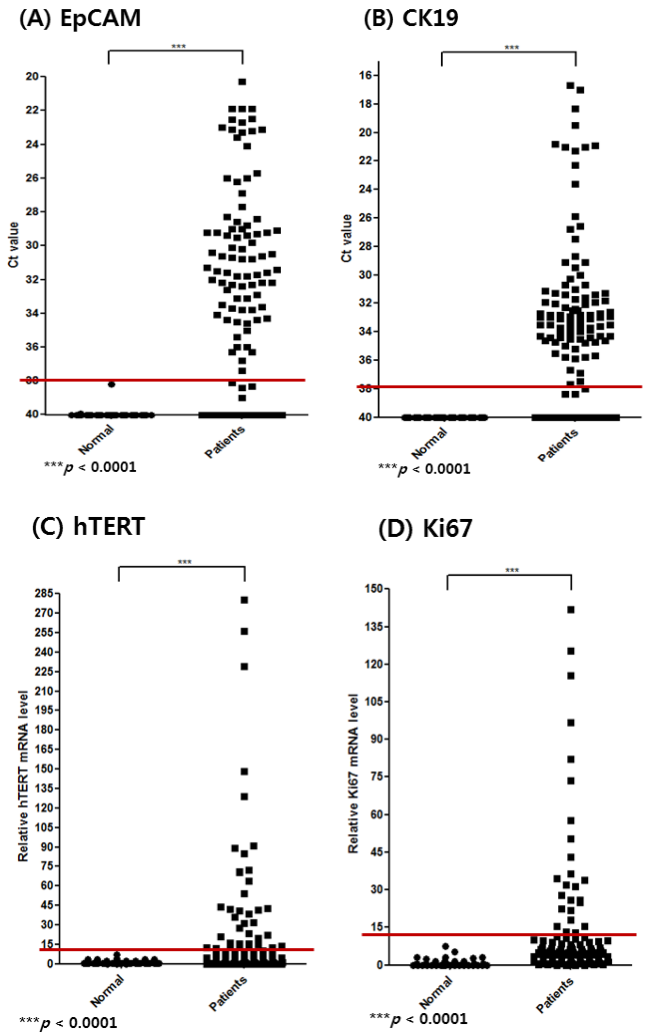


Figure 12. Comparison of the expression of CTC-related markers in the blood of healthy donors and breast cancer patients. In the healthy donor group, the mRNA of all CTC-related markers did not express. In the breast cancer group, the mRNA of CTC-related markers was significantly higher than healthy donors. The *p* values for healthy donors and breast cancer patients were calculated using Student's *t*-test ($p < 0.0001$).

3. Clinical evaluation of HER2 RT-qPCR using blood specimens

In order to confirm the relationship between blood samples showing high levels of HER2 mRNA levels and the samples showing CTC-related markers' expression, blood samples were grouped by HER2 mRNA levels in the blood. As shown in Table 12, the blood samples with high levels of HER2 mRNA either express epithelial markers such as EpCAM/CK19, or proliferation markers such as Ki67 and immortalization markers such as hTERT, indicating HER2 mRNA expressing blood samples may actually contain CTCs.

Table 12. Expression of CTC related markers according to the HER2 status by RT-qPCR

Blood HER2		Epithelial marker	Proliferation marker	n (%)
Positive	39	Positive	Positive	16 (8.5)
			Negative	8 (4.3)
		Negative	Positive	13 (6.9)
			Negative	2 (1.1)
Negative	149	Positive	Positive	14 (7.4)
			Negative	95 (50.5)
		Negative	Positive	5 (2.6)
			Negative	35 (18.6)
Total				**188 (100)**

4. Discordance of HER2 expression between primary tumor and blood

In order to confirm that concordance in HER2 status between primary tumor and CTC in blood, results of HER2 RT-qPCR were grouped by histological HER2 status.

In brief, the results showed that the HER2 mRNA levels in the blood may be quite different from that of the primary tumor (Table 13). Among the HER2-negative primary tumors, HER2 negative primary tumors were found to contain HER2 expressing cells is high with a relatively higher rate in 19.6% of HER2-negative patients (31/158).

The levels of HER2 mRNA expression of these 31 patients were as high as these of HER2 positive. This results show expression of HER2 mRNA in blood and result of histological HER2 examination are different. Therefore, it is evidence that CTC are changed its properties from primary tumor after entering the blood stream.

Table 13. Comparison of HER2 expression detected by HER2 IHC using primary tumor and the HER2 RT-qPCR using blood samples

HER2 status	n (%)
Primary HER2 Positive	**30 (100)**
CTC HER2 (+)	8 (26.7)
CTC HER2 (-)	22 (73.3)
Primary HER2 Negative	**158 (100)**
CTC HER2 (+)	**31 (19.6)**
CTC HER2 (-)	127 (80.4)
Total	188

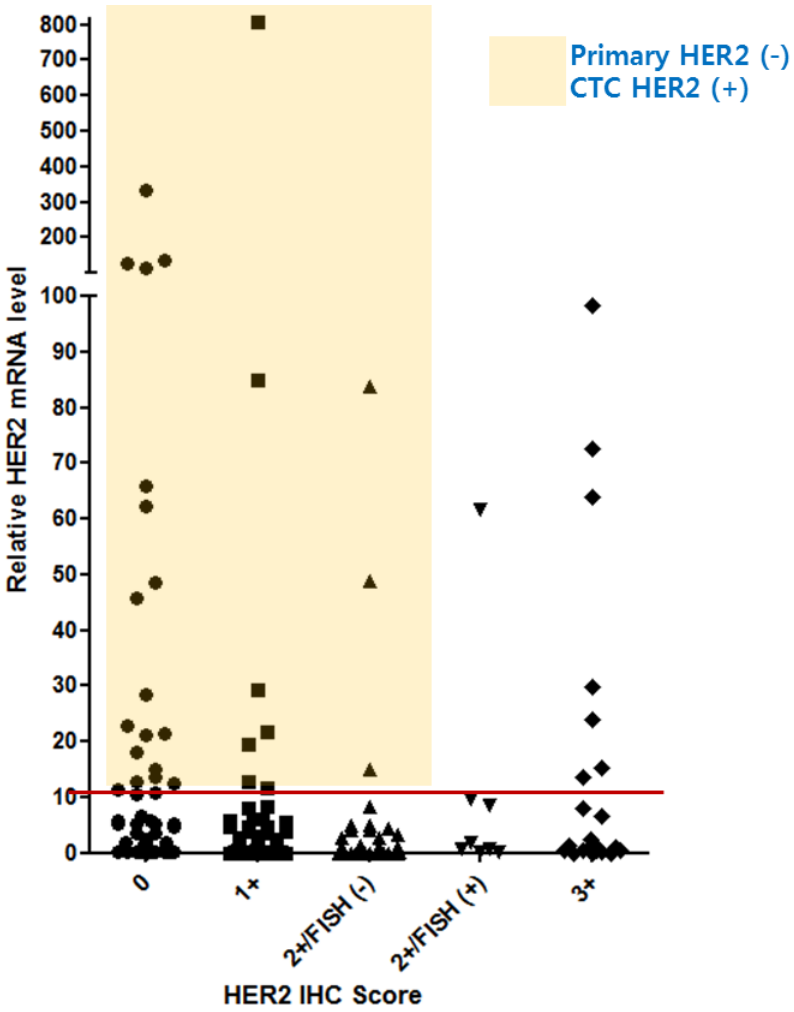


Figure 13. Comparison of the expression HER2 mRNA detected using the blood and histological HER2 status. The mRNA levels of HER2 were analyzed by RT-qPCR using blood samples of breast cancer patients and compared with the histological HER2 IHC test results of each patients.

5. Correlation of cancer association markers mRNA expression with HER2 mRNA expression

In order to confirm that the expression of HER2 mRNA levels are associated with cancer association markers expression, correlation coefficient analysis was performed between HER2 mRNA levels and cancer association markers mRNA levels in blood (Figure 14).

In conclusion, the HER2 mRNA levels in blood had a correlation with Ki67 mRNA levels in blood. (Pearson r=0.4358, R square=0.2360). It was evidence that HER2 over-expression is related with cell proliferation. In addition, HER2 mRNA levels had a correlation with immortalization marker hTERT mRNA levels (Pearson r=0.2988, R square=0.0893). HER2 mRNA levels in the blood were related with cancer cell division and immortalization of cancer cell as well. Therefore, the administration of Herceptin, a targeted therapy for HER2 cancer, to breast cancer patients with a higher rate of CTC HER2 expression is considered to be effective and likely to benefit more breast cancer patients.

However, HER2 mRNA levels in blood did not have any correlation with two epithelial markers.

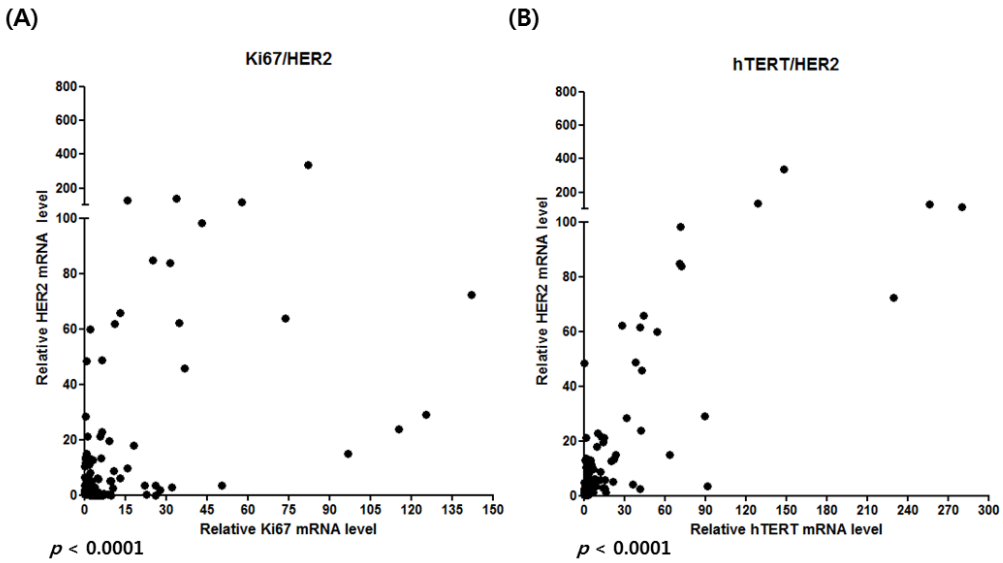


Figure 14. Correlation coefficient analysis between the mRNA levels of Ki67 and HER2 (A), and mRNA levels of hTERT and HER2 (B) in the blood. Correlation between KI67 mRNA level and HER2 mRNA level (r^2=0.2360, p<0.0001) had a correlation. And hTERT mRNA level and HER2 mRNA level (r^2=0.0893, p<0.0001) also had a correlation.

6. Analysis of the CTC related markers in the blood in different breast cancer stages

In order to confirm that expressions of CTC related markers were associated with breast cancer stages, results of RT-qPCR were grouped by breast cancer stages.

As shown in Figure 15,

As the breast cancer stage progress, patients who were over-expressed tumor association markers, such as hTERT, Ki67, and HER2 were tend to increasing.

The number of patients with expression at a ratio of higher than 90 ~ 100 times of mRNA levels increase as the breast cancer stages are progressed. From this result, it is noted that cancer association markers are associated with the stages of breast cancer. The malignancy grade of the breast cancer can be determined by prompt diagnosis with blood testing.

On the other hand, expression of CTC epithelial markers such as EpCAM and CK19 not seem to have correlation (Figure 16).

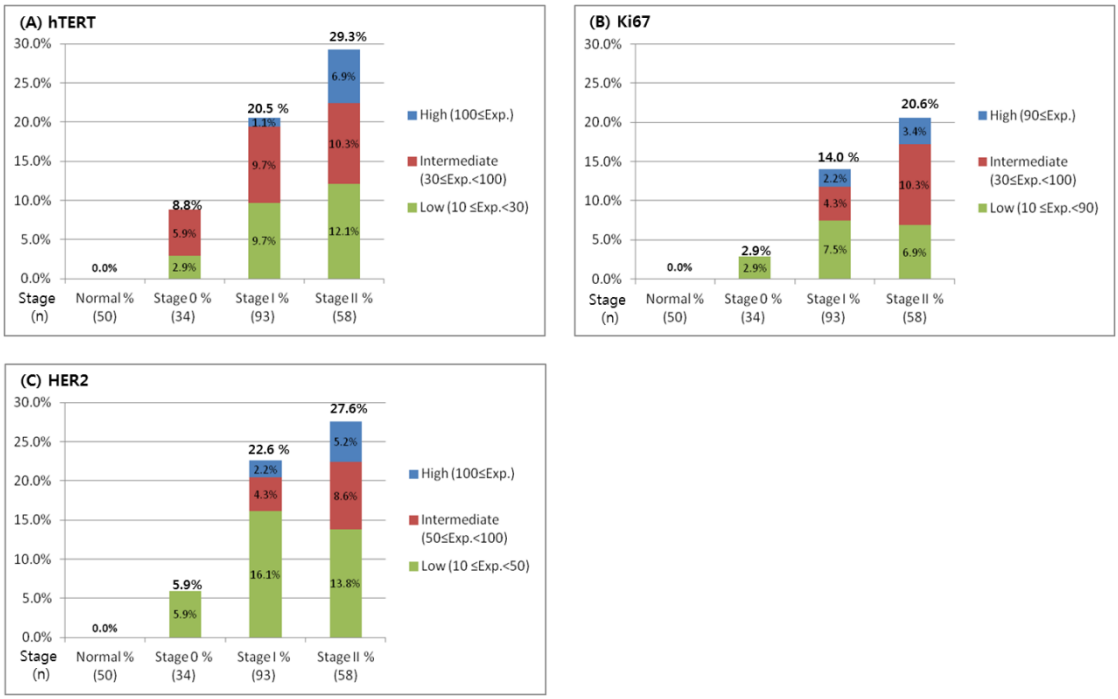


Figure 15. Expression rate of cancer association markers of different breast cancer stages. The all cancer association markers including HER2 were highest mRNA over-expressed in Stage 2 breast cancer. All markers were over-expressed in 20 percent of patients in Stage 2, and ratio of high (expression over 90) expressed patients were more and more increased breast cancer stage became more and more progressed.

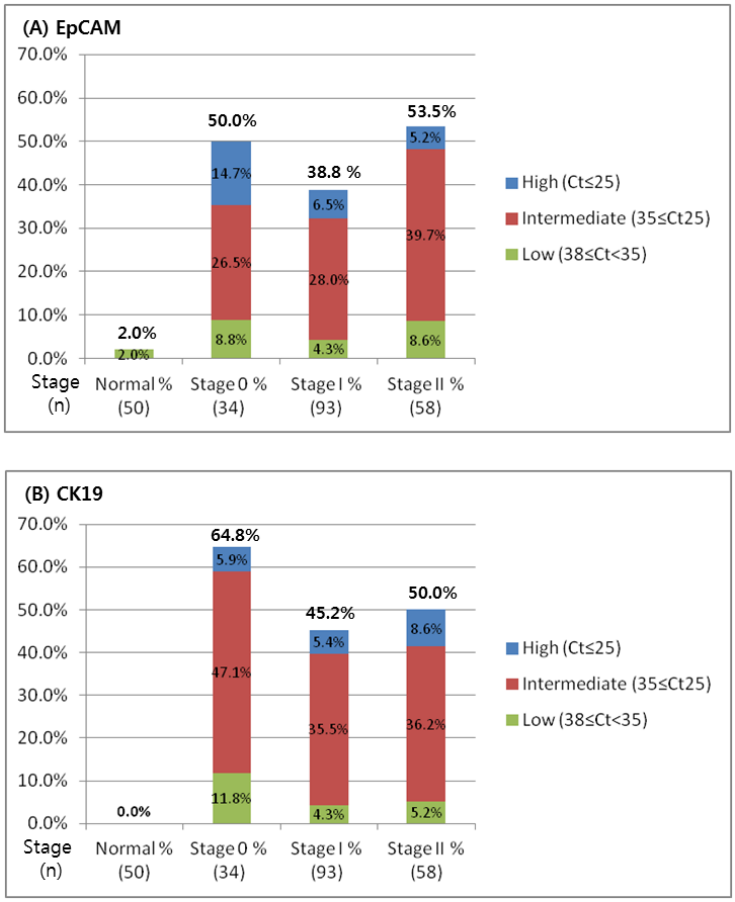


Figure 16. Expression rate of epithelial markers in different breast cancer stages. In the all breast cancer stage including stage 0, about half of patients were high expressed epithelial markers.

IV. DISCUSSION

The presence of disseminated tumor cells in the early phases of primary tumors causes extreme difficulty in the treatment of patients. The presence of CTC in the blood indicates a poor prognosis in patients with breast cancer. In most previous studies, the detection CTC in the blood was done only by determining the presence of CK19 after EpCAM was captured [38,47]. However, it would be valuable to find out the characteristics of the CTC when the treatment breast cancer can be conducted according to the CTC in addition to merely detecting the presence of CTC. In this regard, it will be useful to know whether CTCs in the blood of patients are HER2 positives, since Herceptin is used as a targeted therapy for HER2 cancer. [7,32].

In this study, a molecular diagnosis method was developed based on HER2 RT-qPCR was employed to detects the over-expressing HER2 CTCs in the blood of early-stage breast cancer patients. The analytical sensitivity of HER2 RT-qPCR for detecting HER2 over-expressing CTCs was to the point where only one SK-BR3 cell, a human breast cancer cell line, was able to be detected after it was mixed with 7.5 ml of normal blood (Figure 9). Subsequently, HER2 mRNA expressions in the blood

of a healthy donor group and of breast cancer patients were compared: HER2 mRNA over-expression was not detected in the healthy donor group, whereas 20.7% of the breast cancer patients displayed HER2 mRNA over-expression (Figure 10 and Table 11). HER2 is found to the HER2 over-expression in 20~20% of breast cancer patients. Therefore, the overall sensitivity and specificity of HER RT-qPCR using blood samples seemed to as high as those using biopsy.

In addition, HER2 expression in the blood occurs concurrently with all epithelial markers or cancer association markers in cells. In 37 out of 39 HER2 positive patients, not only HER2 mRNA, but also at least one other type of marker was expressed at the same time. Only two HER2 over-expression patients were HER2 mRNA positive (Table 12). In those patients, it could be possible to increase the detection rate by using more markers in the future.

In this study, it is demonstrated that CTC HER2 expression may be quite different from that of the primary tumor. Previous studies have demonstrated that expression of HER2 molecules on CTCs was independent of its expression on the primary tumor cells [52-54]. The reasons for the discrepancy of HER2 status

between occult tumor cells and primary tumor cells are not obvious. It has been proposed that these cells may represent an early disseminated subclone of HER2-positive cells of the primary tumor, which is microscopically undetectable because of their very low frequency. Previous studies have demonstrated that cytokeratin/HER2 double positive clustered peripheral blood cells have a high potential for locomotion suggesting that they might be precursor cells responsible for the development of CTCs [54]. In addition, it has been shown that HER2 gene amplification can be acquired during breast cancer progression [55]. This hypothesis is further supported by the previous reported 14% discordance of HER2 over-expression between the primary tumor and the corresponding metastases in patients with breast cancer [12].

Despite all the above evidence, the data from this study should be interpreted with caution since different methods have been used to detect HER2 expression in primary tumor cells and HER2 expression in blood.

Breast cancer patients who are HER2 positive in the primary tumor are treated with Herceptin. Breast cancer patients who are HER2 negative in primary tumor will need other treatments. But,

HER2 mRNA over-expression was observed in the blood of 31 out of 158 (19.6%) patients who were HER2-negative for the primary tumor (Figure 13 and Table 13). These patients are not currently given Herceptin, but the administration of Herceptin is likely to be effective and more breast cancer patients will benefit through it. Therefore, these patients who had a potential of metastasis were more effective management for breast cancer prognosis.

It is noted that the HER2 mRNA expression in the blood is associated with cancer association marker expression, hTERT and Ki67 (Figure 14). This shows a highly significant correlation between HER2 mRNA expression and the malignant progression of tumors and/or immortal cancer cells. In addition, rate of patients who are over-expressing cancer association markers is increasing as breast cancer is more progressed (Figure 15). Therefore, the results from this study seems to suggest that demonstrate in the case of CTC in the blood through the administration of Herceptin for HER2 positive CTC patients, is a very effective for breast cancer patients resulting in a good prognosis.

In this study, a “liquid biopsy,” blood is used because it

provides a relatively easy and noninvasive way to sample tumors. Therefore, it is expected to allow for more effective management of and a better prognosis for breast cancer. Prospective trials targeting the residual HER2 mRNA positive tumor cells in patients with early stage breast cancer could demonstrate the importance of a “Secondary” adjuvant therapeutic approach.

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국문요약

실시간역전사중합효소연쇄반응을 이용한 유방암 환자 치료제 선별을 위한 분자진단검사법 개발

여성의 주요 사망원인인 유방암을 치료하기에 앞서 Human epidermal growth factor receptor 2 (HER2)와 Estrogen receptor (ER), Progesterone receptor (PR) 단백질이 발현하고 있는지 여부를 검사하는 것은 매우 중요하다. 왜냐하면 HER2가 양성인 유방암에는 Herpcetin을 이용한 표적치료가 가능하고, ER이나 PR이 양성인 유방암에는 Tamoxifen과 같은 Estrogen 억제제를 이용한 표적치료가 가능하여 환자의 치료효과를 높일 수 있기 때문이다. 현재 HER2, ER, PR 검사에는 면역조직화학염색법과 형광동소보합법을 사용하고 있다. 그러나 이 검사법들은 현미경으로 염색결과를 판독해야 하는 특성으로 인하여 검사과정이나 결과의 판독이 정략적으로 표준화하기 어렵고 실시간 검

사가 곤란하다는 단점을 가지고 있다. 따라서 본 연구에서는 HER2, ER, PR의 발현여부를 표준화된 검사과정을 통해 정량적인 검사결과를 제시할 수 있는 실시간역전사 중합효소연쇄반응 (RT-qPCR) 검사법을 개발하였고, 환자의 조직 검체를 대상으로 개발한 새로운 검사법을 평가하였다. 그 결과 본 연구에서 개발한 HER2 검사법의 민감도와 특이도는 각각 93.0%와 89.1% 였고, ER과 PR 동시검사용 방법의 민감도와 특이도는 각각 80.2%와 94.8%였다. 또한 면역조직화학염색법과 형광동소보합법의 또 다른 문제점은 조직을 이용한 침습적인 검사법이기 때문에 지속적인 환자 예후를 관찰하기 위한 검사법으로 적절하지 않다는 점이다. 그러나, RT-qPCR을 이용한 검사법은 유방암 환자의 혈액 내에 존재하는 혈중암세포를 이용하여 환자의 예후 관찰 및 실시간 검사가 가능하므로 본 연구에서는 혈액검체를 이용한 HER2 RT-qPCR 평가하였다. 정상인의 혈액검체를 대상으로 한 검사에서는 과량의 HER2 mRNA가 검출 되지 않은 반면, 유방암 환자의 20.1%에서는 과량의 HER2 mRNA를 확인하였다. 또한 과량의 HER2 mRNA가 포함된 검체는 혈중암 세포 마커인

EpCAM, CK19, Ki67, hTERT 중 적어도 하나 이상을 동시에 발현하고 있어 특정 암세포에 의해 HER2 mRNA가 과량 만들어짐을 유추할 수 있었다. 한편 조직을 이용한 기존의 HER2 검사법과 혈액을 이용한 HER2 RT-qPCR 검사가 반드시 일치 하지 않는 것을 확인 할 수 있었고, 이는 고형암 상태의 유방암이 혈액 내로 들어오게 되면서 그 상태가 변화할 수 있음을 의미하는 것으로 생각할 수 있다. 따라서 혈액 HER2 검사에서 HER2 mRNA가 과량 검출된 환자에게 Herceptin 투여를 고려해 볼 가능성을 보여준다. 이러한 RT-qPCR을 이용한 검사법은 표준화된 검사법을 검사실마다 제공 할 수 있는 좋은 검사법이 될 수 있고, 뿐만 아니라 유방암 환자의 치료 및 예후 관리에 있어서 도움을 줄 수 있을 것이라고 생각된다.

핵심되는 말 : HER2, HR, RT-qPCR, Herceptin, Tamoxifen, 표적치료, 순환종양세포