Development of Gel-based Multiplex RT-PCR for Detection of ER/PR/HER2-Positive Breast Cancer Diagnosis

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ABSTRACT: Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression levels play a central role as prognostic and predictive markers in breast cancer specimens. Therefore, detecting ER, PR, and HER2 statuses is essential for determining a correct therapeutic method to treat breast cancer. The most commonly used assays in clinical studies for detecting the expression levels of these genes are immunohistochemistry (IHC) and fluorescence in situhybridization (FISH). However, HC and FISH frequently underestimate ER, PR, and HER2 levels. A multiplex quantitative and cost-effective assay using gel-based reverse transcription-polymerase chain reaction (RT-PCR) for the assessment of ER, PR, and HER2 was developed to overcome this problem. Multiplex RT-PCR provided consistent data in four breast cancer cell lines without any cross-amplification of cDNA from other genes indicating that the developed assay was reliable in its specificity. In fact, this multiplex RT-PCR assay proved to be a sensitive and convenient method to rapidly and simultaneously detect the expression levels of ER, PR, HER2, and Pumilio homolog 1 (PUM1). In conclusion, multiplex RT-PCR could be useful for routine diagnosis of ER-, PR-, and HER2-positive breast cancers.

KEYWORDS: Biology; Cancer Biology; Breast Cancer; Gene Expression; RT-PCR.

INTRODUCTION

The advancement and widespread application of genomics, transcriptomics, and proteomics has provided novel understanding of breast cancer's molecular complexity.[1] However, clinical decisions still rely on the assessment of three molecular markers: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2).[2] Identifying these markers is essential for efficient targeted treatment for different types of breast cancer.[2]

ER is activated by estrogen.[3] There are two types of ER: ERα and ERβ.[4] Once activated, ERα and ERβ form dimers and translocate into the nucleus to facilitate the regulation of various genes.[4] Approximately 80% of breast cancer patients display ER-positive breast cancer.[5] ER-positive breast cancertypically responds readily to hormone-targeted therapy.[6] ER-positive cancers are treated with tamoxifen, a drug that blocks hormone receptors and the inhibition of estrogen production.[7]

PR is a receptor that blocks transcription until activated by progesterone.[8] Approximately 65% of breast cancer patients display PR positive breast cancer.[9] While PR may not respond to endocrine therapy directly, its activation may have significant impacts on the ER signaling pathway, thus showing value in determining which tumors may be subject to PR reprogramming of ER.[10]

HER2 is a breast cancer diagnostic marker that aids the therapeutic decisions in the treatment of breast cancer.[11,12] Up to 30% of patients display HER2 positive breast cancer.[13] HER2 gene amplification in breast cancer cells is linked to a more clinically aggressive response in patients and corresponds to a higher death rate.[14,15] HER2 positive breast cancer is normally treated with trastuzumab, a monoclonal antibody that induces the downregulation and internalization of HER2 as well as upregulates cell cycle inhibitors.[16,17]

Previously, detection of ER, PR, and HER2 in breast cancercells had been widely done using the immunohistochemistry (IHC) and fluorescence in *situ* hybridization (FISH) methods.[18,19] However, immunostaining methods like IHC are naturally prone to errors as the results are semi-quantitative and subject to interobserver variability.[20] The specificity of IHC depends on the quality of antibodies because IHC involves the process of selectively identifying antigens in cells of a tissue section.[21] Thus, these methods often lead to false positive/negative results. FISH is often used for diagnostic decisions by determining the ratio of the number of signals from two different chromosomal regions to determine gene amplification.[22] FISH assessments deliver more sensitive and quantitative results but is expensive and time-consuming, thus not appropriate for widespread use.[23] Furthermore, FISH-methods are prone to ambiguous results as standard protocols for sample collection and storage have not been developed.[23]

Detection of receptor DNA or cDNA by PCR meets thecriteria of speed and high sensitivity and is used frequently for diagnostic purposes.[24] Gel-based reverse transcription polymerase chain reaction (RT-PCR) assay was reportedly

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successful in the diagnosis of African swine fever (ASF).[25] This assay is sensitive and specific for the fast and early diagnosis of ASF. Therefore, RT-PCR could be an alternative method for the detection of ER, PR, and HER2.[26] Furthermore, multiplex PCR allows for simultaneous amplification of multiple target sequences in a single tube using specific primer sets in combination.[24,27] Thus, multiplex PCR favors higher throughput and automation compared to singleplex PCR especially when testing repeated and numerous patient analyses of the same targets.[28] To overcome the problems of two-step real time singleplex RT-PCR, a highly sensitive and specific gel-based multiplex RT-PCR assay was developed in this study by testing ER, PR, and HER2 negative and positive breast cancer cell lines.

RESULTS

With the molecular classifications of breast cancer, researchers focus on breast cancer cell lines to determine whether the molecular profiles observed in breast cancer patients are reflected in cell line models of the disease.[18] Applications of transcriptional profiling to breast cancer cell lines using various platforms provided the cell lines' characteristics by the expression of estrogen receptor (ERβ), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).[29] Four cell lines were selected to observe ER, PR, and HER2 expressions with RT-PCR method (Table 1).

Table 1. Categorization and molecular information of four breast cancer cell lines and the clinical features of tumors where they are derived. AC is "adenocarcinoma" and IDC is "invasive ductal carcinoma."

Cell lines	ER	PR	HER2	Tumor
MCF7	+	+	-	IDC
MDAMB231	-	-	-	AC
MDAMB453	-	-	+	AC
BT474	+	+	+	IDC

A previous study indicated that PUM1 is one of the best housekeeping genes for normalization of gene expression levels in both ER positive and negative subgroups and in normal breast tissue.[30] We designed the primer sets for ER, PR, HER2, and PUM1 at different lengths so they could be differentiated during agarose gel electrophoresis (Table 2).

The PCR reaction is visualized using agarose gel electrophoresis. DNA fragments of the expected size of amplified HER2, ERβ, PR, and PUM1 form a strong amplified DNA band on the gel by singleplex RT-PCR in all four breast cancer cell lines (Figure 1). Even though PCR conditions were optimized to amplify the target genes, some nonspecific bands were detected. PCR conditions were not perfectly optimized but the primers were sensitive enough to amplify the targeted gene.

Table 2. Sequence information for primers used in RT-PCR.

Gene	Forward Primer	Reverse Primer	Expected Size (bp)	Targeted Exon
HER2	GAAGGTGAAGGTGCTTGGATCTGG	TAGCTCATCCCCTTGGCAATCTGC	342	Exon 18-20
ER-beta	TCCTCCTACAACTGCAGTCA	ACTGGCGATGGACCACTAAA	244	Exon 2 only
PR	ATGGAAGGGCAGCACAACTA	AGGGCTTGGCTTTCATTTGG	224	Exon 2-4
PUM1	TGAGGTGTGCACCATGAAC	CAGAATGTGCTTGCCATAGG	187	Exon 21-22

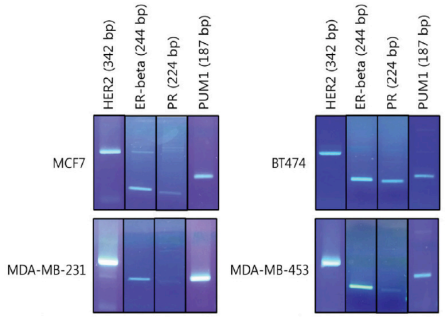

Figure 1. Agarose gel electrophoresis of singleplex RT-PCR products from cDNA of MCF7, BT-474, MDA-MB-231, and MDA-MB-453 breast cancer cell line.

Some nonspecific bands showed in Figure 1 could bea result of residual genomic DNA from the RNA extraction process because control reactions were not performed in this experiment.

Since the PCR conditions were optimized in Figure 1, multiplex RT-PCR was performed in four different breast cancer cell lines. Multiplex RT-PCR showed viable results for all cell lines as the relative expression levels for ER, PR, and HER2 were low for the ER, PR, and HER2-negative breast cancer cell lines and high for the ER, PR, and HER2-positive breast cancer cell lines (Figure 2). PUM1 also proved to be very compatible with ER, PR, and HER2 in the multiplex process as the multiplex results yielded consistent expression levels in breast cancer cell lines (Figure 2). These observations show that gel-based RT-PCR multiplex detection of ER, PR, and HER2 is a reliable way of detecting the expression of ER, PR, and HER2 in breast cancer cell lines and have the potential to be further applied to other genes involved in breast cancer.

CellExpress is a web-based tool that allows for analysis of gene expression levels in all of the cancer cell lines and clinical samples available online. The program takes queries based on gene, cell line, and normalization method and outputs all relevant data with a value corresponding to the relative expression of the gene and microRNA in the cell line in question.[31] This system is highly useful for cross analysis of gene expression in different cell lines, as it provides a universal ranking and value with the same normalization method.

Figure 3 shows the relative expression of the four studied genes in the MCF7, BT-474, MDA-MB-231, and MDAMB-453 cell lines. PUM1 has a uniform distribution of relative expression and low standard deviations as consistent with pre-

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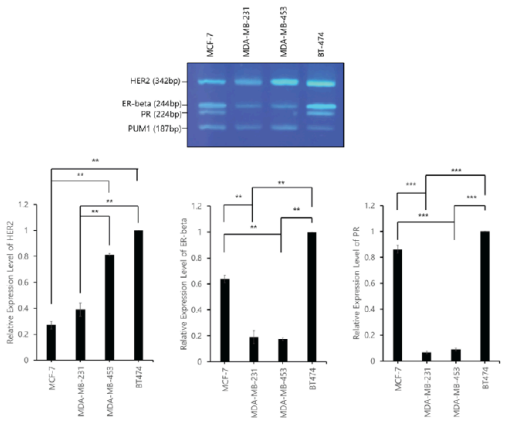

Figure 2. Quantification of HER2, ERβ, and PR expression level on cDNA from MCF7, BT-474, MDA-MB-231, and MDA-MB-453 using agarose gel electrophoresis of multiplex RT-PCR. (upper) Gel electrophoresis of HER2 (342bp), ERβ (244bp), PR (224bp), and PUM1 (187bp) (lower) Bar chart of band intensity calculated for HER2 expression level. The expression level of HER2, ERβ, and PR was normalized by PUM1 expression level (Mean + SD). Student's t test, ** P<0.01, *** P<0.001.

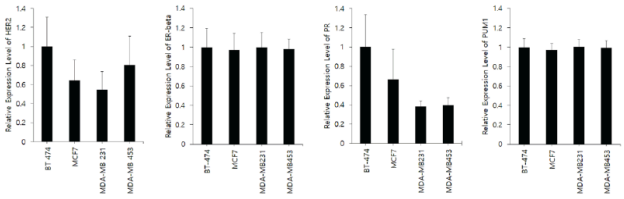

Figure 3. Relative expression for HER2, ERβ, PR, and PUM1 from MCF7(n=6), BT-474(n=6), MDA-MB-231(n=6), and MDA-MB-453(n=6) using cell line microarray data provided by CellExpress (Mean + SD).

vious studies, suggesting it is an appropriate normalizing gene for this study.[30]

All microarray data is consistent with Figure 2 except the relative expression of ERβ. The microarray data shows a much more even distribution of expression of ERβ compared with Figure 2, which shows distinct differences in expression between the four cell lines. This could be explained by the fact that ERβ has nine exons in total and the selected region for the microarray and the primer could have targeted different exons in the gene. Since there are several isoforms of ERβ the relative expression could differ depending on targeted exon.

Triple negative breast cancer is more likely to metastasize which further emphasizes the need for fast detection of receptor positive/negative cancer.[32] To visualize the effects of triple negative breast cancer on patients, 14 studies and 9134 samples were compiled to construct data (Figure 4) using a website called cBioPortal to analyze the difference in survival rate between patients with gene deletion in HER2, ER, and/or PR and patients without the deletion.[33] There was a markedly lower rate of survival in patients with gene deletion with a p-value of 1.237e-3. A previous study on a Brazilian cohort confirms that Triple Negative Breast Cancer (TNBC) displays a more aggressive behavior, recurs more frequently, and has a worse survival rate.[34] These patients have to receive different treatment (i.e. chemotherapy) and make it crucial to detect the expression of these receptors in the early stages of cancer.[32]

Discussion

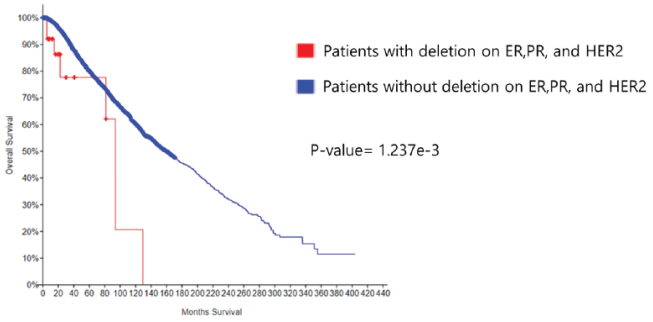

Figure 4. The overall survival rate (Kaplan-Meier survival curve, Log-rank test, p-value = 1.237e-3) between patients with gene deletion in HER2, ER, and PR and patients without the deletion using cBioPortal database (14 studies with 9134 patient samples)

FISH and IHC are widely accepted as the best methods for ER, PR, and HER2 detection, a critical factor in the treatment of invasive breast cancer patients.[18,22] However, these methods both have limitations and are not perfect at detecting ER, PR, and HER2.[35] RT-qPCR detection of ER, PR, and HER2 has many advantages over these two methods, including cost timeliness, accuracy, and sensitivity.[36] The results in this study show that the gelbased RT-PCR assessments were accurate in detecting the amplification of ER, PR, and HER2 in different breast cancer cell lines (Figure 2). The results from this study are consistent with various microarray data (Figure 3). Which further confirms the accuracy of this method. Therefore, ER, PR, and HER2 detection via gelbased RT-PCR can remediate the disadvantages of methods like IHC and FISH in the future for rapid and accurate detection of ER, PR, and HER2 in a large number of samples. Since ER, PR, and HER2 expression levels are quantitatively measured by gelbased multiplex RT-PCR, measuring the expression level of novel genes relate to breast cancer progression by gelbased multiplex can be developed in the future.

A limitation of this study is that the qRT-PCR was tested on breast cancer cell lines instead of tissue samples from actual patients. Cancer cell lines were initially derived from tumors and cultured in two dimensional conditions. Cell cultures are widely used as models to study molecular markers of cancer.[37] However, data obtained from the cancer cell lines could be different from the data obtained from actual patient tissue samples as tissue samples are often heterogeneous, containing tumor cells as well as normal cells such as red blood cells.[38] Previous research found a strong correlation of the expression levels of ER (r=0.85) and PR (r=0.9) between IHC and RT-qPCR methods in patient tissue samples.[39] This result suggests that qRT-PCR is a promising complementary method to IHC for determining hormone receptors and protein markers used in breast cancer diagnosis.[39]

All primer pairs have an unknown amplification efficiency so they may differ in their ability to anneal and promote amplifi

cation of their target DNA region.[40] The primers used in this study were not tested to prove their equal efficiency to anneal the target gene to promote DNA amplification so the expression level of one marker gene relative to another in each cell lines could be inaccurate. Therefore, the annealing ability of primers should be analyzed to further address this limitation.

Development of a multiplex RT-PCR assay for four different genes required careful selection of primers and manipulation of PCR conditions because multiple primers could dimerize or suppress other primers. In each gene there are several isoforms that consist of different combinations of exons.[41-43] Thus, primers are chosen from a specific gene region that is conserved in most isoforms to eliminate biases related to uneven isoform representation. Ensembl, a web-based genome browser, was employed to visualize the exon structure and sequence of the four genes and exon regions.[44] Furthermore, we ensured the melting temperature of the primers were similar and differentiated the length of PCR products to ensure distinct detection during gel electrophoresis. Primers were tested individually to ensure optimal PCR conditions as well as in different combinations of over 500 PCR reactions to maximize the multi-PCR products' quality.

CONCLUSION

The result of this study will help for rapid detection of the expression level of ER, PR, and HER2, especially in laboratories that cannot afford a real-time PCR machine. Unlike FISH and IHC assays, our assay is much simpler and more convenient. The multiplex analysis greatly reduces time-consuming procedures and eliminates additional manipulations that FISH and IHC assays require.

METHODS

Breast Cancer Cell Lines

All cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were maintained in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Thermo Fisher) and 1% penicillin and streptomycin in a 5% CO2 atmosphere at 37°C. All cells were kept in culture for four or fewer passages and cell phenotypes were verified in every experiment. Four different known breast cancer cell lines were used in this study as templates for the HER2 detection: MCF7, MDAMB231, BT474, and MDAMB453. These cell lines display different types of cell markers being HER2-, triple negative, triple positive, and HER2+ respectively (Table 1).

Primer Set Design

Primers were designed for ER, PR,HER2, and PUM1 with GenomCompiler program and they were synthesized by Bioneer (Table 2). The amplified DNA products were designed to be different lengths to be differentiated during agarose gel electrophoresis with ER (244 bp), PR (224 bp), HER2 (342 bp), and PUM1 (187bp). The annealing temperature was set for 60 °C for downstream applications.

RT-PCR Reaction

RNA was extracted from breast cancer cell lines by RNAspin™ Total RNA Extraction Kit (Intron) following the manufacturer's instructions. cDNA was synthesized from the extracted RNA using TOPscript™ Reverse Transcriptase (Enzynomics) following the manufacturer's instructions. For the PCR reaction, the different primers were tested individually using 20 μL reaction containing 2μL forward/ reverse primers (10 pmol), 2μL reaction buffer, 2μL dNTP, 1.125μL Taq polymerase(Bioneer), 0.5 μL cDNA, and, RNase Free dH2O up to 20 μL. PCR was done with an annealing temperature of 60°C and an extension time of 40 seconds in 72°C for 40 cycles. For the multiplex reactions, nTaq-multi HOT (Enzynomics) was used with 20 μL reaction containing 2μL forward/reverse primers, 0.2μL nTaq- multiHOT polymerase, 0.5 μL cDNA, and RNase Free dH2O up to 20 μL.

Agarose Gel Electrophoresis

2-5% agarose gel and TBE buffer were used in this study. RedSafe™ Nucleic Acid Staining Solution (Intron), an alternative to traditional ethidium bromide (EtBr), was used to stain nucleic acid. Amplified DNA was detected by direct examination of the gel in ultraviolet (UV) light.

Agarose Gel Quantification Analysis

LI-COR Image Studio software version 2.1.10 and gel images were saved as a work area. All gel intensity quantification analyses were performed on images saved in TIFF format.

All experiments in this study, including running the agarosegel electrophoresis, were performed in University of Suwon, Korea.

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