

Detection of circulating tumor cells in patients with breast cancer using the conditionally reprogrammed cell culture method and reverse transcription-PCR of *hTERT* and *MAGE A1-6*

YOUNG JU JEONG¹, SUNG HWAN PARK¹ and CHANG-HO JEON²

Departments of ¹Surgery and ²Laboratory Medicine, Catholic University of Daegu School of Medicine, Daegu 42472, Republic of Korea

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Abstract. The present study aimed to verify the efficacy of the conditionally reprogrammed cell (CRC) culture method for the detection of circulating tumor cells (CTCs) in breast cancer. CTCs were isolated from the peripheral blood of patients with breast cancer, and culture of the collected CTCs was performed according to the conditional reprogramming protocol. Total RNA was extracted from cultured CTCs, and the *hTERT* and *MAGE A1-6* genes were amplified using reverse transcription-PCR (RT-PCR). In addition, RNA extraction from another blood sample was performed and the expression of the two genes was analyzed by RT-PCR only. Following CRC culture, grown CTCs were observed in 7 samples (23.3%). The CTC detection rates by RT-PCR for the *hTERT* and *MAGE A1-6* genes in CTCs grown using the CRC culture method were 26.7 and 10.0%, respectively. The positive expression rates for the *hTERT* and *MAGE* genes in CTCs assessed by RT-PCR only were 44.1 and 23.5%, respectively. When combining the positive expression rates of RT-PCR only and CRC culture for the *hTERT* and *MAGE A1-6* genes, CTC detection rates increased to 53.3 and 23.3%, respectively. Additionally, when combining the positive expression rates of the two genes by either method, the CTC detection rate was the highest value observed. In conclusion, the present study revealed the potential of CRC culture in the detection of CTCs in breast cancer. Furthermore, a combination of CRC culture and RT-PCR for the *hTERT* and *MAGE A1-6* genes is useful in enhancing the detection rate of CTCs in the blood.

Introduction

Liquid biopsy is often used as a technique for detecting biomarkers in body fluids, including blood, urine, saliva, pleural effusions, ascites and cerebrospinal fluid (1). Human body fluids contain biologic materials such as cell-free DNA and RNA, proteins, cells and vesicles (1). These materials can be representative of the tissues from which they originated, and thus liquid biopsy has the potential to be used instead of tissue-based biopsy in clinical practice and for research purposes. In addition, liquid biopsy allows clinicians to perform biopsies repeatedly and non-invasively.

Circulating tumor cells (CTCs) are tumor cells derived from primary tumors and/or metastatic lesions, and circulate freely in peripheral blood (2). CTCs have characteristics specific to their tumor of origin (2) and can migrate to the circulatory system causing metastasis (3). Recently, many studies have described the potential applications of CTCs in the diagnosis, evaluation of treatment response, and targeted therapy of cancer (4-8).

Multiple technologies have been developed to detect and isolate CTCs, but quality detection of CTCs remains a central problem in the study of CTCs. Most detection methods are based on the detection of epithelial cell adhesion molecule (EpCAM), cytokeratin, and lack of the leukocyte common antigen CD45 (3). Furthermore, CTCs can be sorted based on physical characteristics such as size, density, deformability, and electrical charge (1). The CellSearch[®] system is an automated EpCAM-based system and is the only technology approved by the US Food and Drug Administration in a detection of CTCs for the clinical use (3). Although the CellSearch[®] system is considered the standard method for the detection of CTCs, several issues still exist regarding this system. Not all CTCs express EpCAM (2), and CTCs are heterogeneous among cancer type and even within individuals (3). Moreover, CTCs isolated by the CellSearch[®] system are fixed and unavailable for functional analysis (9). Thus, there is a need to develop more specific and efficient methods to distinguish viable CTCs from other cells.

The melanoma antigen encoding gene (*MAGE*) family is known to encode tumor-specific antigenic peptides (10) and is dysregulated in many cancers (11), and the human telomerase

Correspondence to: Professor Chang-Ho Jeon, Department of Laboratory Medicine, Catholic University of Daegu School of Medicine, 33 Duryugongwon-ro 17-gil, Nam-gu, Daegu 42472, Republic of Korea
E-mail: chjeon@cu.ac.kr

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reverse transcriptase (*hTERT*) gene is also known to be expressed in most cancer cells (12). In our previous study, we used reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of the *MAGE A1-6* and the *hTERT* genes to detect CTCs in blood, and our results showed good sensitivity and specificity (13).

It has been suggested that *ex vivo* culture and characterization of CTCs may be utilized in the study of cancer metastasis and patient-derived tumor models (7,9), and in recent years, several studies have shown the results of CTCs culture in different culture conditions (7,14-17). Liu *et al* (18) showed that small numbers of cells can be cultured effectively by generating conditionally reprogrammed cell (CRC) (18). Conditional reprogramming (CR) is a cell culture technique culturing patient-derived cells with feeder cells and a Rho kinase (ROCK) inhibitor (18). Recently, Zheng *et al* (19) investigated CRC culture method that is efficient for the culture of CTCs *in vitro* and showed that the cultured cells preserve their original phenotype. Detection of CTCs using CRC cultures appears to be a very efficient alternative method, but a significant amount of research is still required.

In this study, we aimed to verify the efficacy of a CRC culture method for the detection of CTCs. Further, to enhance the detection rate of CTCs, we utilized a joint method combining CRC culture with RT-PCR of the *MAGE A1-6* and the *hTERT* genes in blood.

Materials and methods

Patients and specimens. A total of 34 patients with breast cancer who had undergone surgery at Daegu Catholic University Hospital (Daegu, Korea) were included. To detect CTCs and analyze RNA levels of target genes, two 6-ml samples of peripheral blood were drawn into ethylenediaminetetraacetic acid (EDTA) coated tubes from each patient before surgery. Specimens were stored at 4°C until further analysis. All data were recorded prospectively, and the clinicopathologic characteristics of the patients were evaluated from medical records. Cancer staging was assessed according to the 7th edition of the American Joint Committee on Cancer staging manual for breast cancer. Written informed consent was obtained from all patients according to the protocol approved by the Institutional Review Board of the School of Medicine, Catholic University of Daegu.

CTCs isolation. To detect CTCs, we collected peripheral blood mononuclear cells (PBMC) after red blood cells (RBCs) lysis from the blood. CTCs were isolated by negative immunomagnetic separation technology (20) using the CD45 antibody capture system as previously described (13). Briefly, the RBCs were lysed with RBC lysis buffer (Roche Diagnostics GmbH). Following RBC lysis, the cells were resuspended in 80 μ l of CD45 binding buffer and reacted with 20 μ l of microbeads conjugated to monoclonal antihuman CD45 antibodies (cat. no. 130 045 801; Miltenyi Biotec GmbH) for 15 min at 4°C. The CD45⁺ cells were captured using a magnetic separator, and the CD45 cells were eluted and collected.

RNA extraction. Total RNA of PBMCs was extracted from one blood sample using the RNeasy Mini kit (Qiagen AB), or

TRIzol™ reagent (cat. no. 15596026; Invitrogen (Carlsbad); Thermo Fisher Scientific, Inc.) and TRIzol Plus kits (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. Briefly, the samples were lysed and homogenized in 1 ml of TRIzol™ reagent, and then incubated for 5 min at room temperature. Chloroform (0.2 ml) was added to the samples in TRIzol™ reagent, and incubated for 2-3 min at room temperature. After centrifugation for 5 min at 12,000 x g at 4°C, RNA in the samples was precipitated by adding isopropanol. The samples were washed with 1 ml of 75% ethanol and the supernatant was discarded after the centrifugation. RNA was then dissolved with RNase free water and 0.5% SDS solution. RNA yield was determined by measuring absorbance at 260 and 280 nm.

Reverse transcription polymerase chain reaction (RT PCR). RNA extracted from PBMC was then amplified for the *hTERT* and the *MAGE A1-6* genes following the method described in previous studies (13,21). Briefly, extracted RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega Corporation) according to the manufacturer's protocol. The *hTERT* and the *MAGE A1-6* genes were amplified using the LightCycler FastStart DNA Master System (Roche Diagnostics GmbH) according to the manufacturer's instruction, and detection was performed using the LightCycler 2.0 (Roche Diagnostics GmbH). The *GAPDH* gene was used as a housekeeping gene for normalization. Each experiment was independently repeated at least three times.

CRC culture of CTCs. From the remaining blood sample, we collected CTCs and cultured them according to the CR protocol (22) for 4 weeks. Before CRC culture of CTCs in patients' blood samples, a reproducibility experiment of CRC culture was performed using a cell line. According to the CRC culture method, CR cell lines are generated from tissue samples using co-culture with irradiated J2 feeder cells (irradiated Swiss 3T3 J2 mouse fibroblast cells) and Y-27632 (ROCK inhibitor) (22). Briefly, before co-culture, Swiss-3T3-J2 mouse fibroblasts were cultured to generate feeder cells and then isolated CTCs were plated in F medium containing J2 feeder cells and Y-27632 (cat. no. 270-333M025, Enzo Life Sciences, Lausen, Switzerland). F medium was made by mixing Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gaithersburg, MD), 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), l-glutamine (Gibco; Thermo Fisher Scientific, Inc.), penicillin/streptomycin mix (Gibco), F12 nutrient mix (cat. no. 11765-054, Gibco), 25 ng/ml hydrocortisone (Sigma-Aldrich), 0.125 ng/ml epidermal growth factor (EGF) (Invitrogen; Thermo Fisher Scientific, Inc.), 5 μ g/ml insulin (Sigma-Aldrich), 250 ng/ml amphotericin B (Thermo Fisher Scientific), 10 μ g/ml gentamicin (Gibco; Thermo Fisher Scientific, Inc.) and 0.1 nM cholera toxin (Sigma-Aldrich). All cells were maintained at 37°C in a cell culture incubator with 95% humidity and 5% CO₂. The experiment was independently repeated at least three times.

We identified cells grown in the culture plates and the cells were defined as CTCs grown by CRC culture, because the lifespan of CTCs is short and most of CTCs cannot survive

for a long time after isolation, and would only be present if they were able to grow in culture condition. Following culture of CTCs by CRC culture methods, culture plates were treated with 1ml of TRIzol™ reagent and total RNA was extracted as above. Then, the *hTERT* and the *MAGE A1-6* genes were amplified and analyzed using RNA extracted from cultured CTCs.

Statistical analysis. Statistical analysis was performed using SPSS software (version 15.0; SPSS, Inc.). CTCs were defined as cells isolated by negative immunomagnetic separation with positive expression of the *MAGE A1-6* or the *hTERT* gene. The detection rate was defined as the percentage of samples with positive CTCs among all samples included in the analysis. The gene expression rates were defined as the percentage of samples with positive expression of the *MAGE A1-6* or the *hTERT* gene among all samples included in the analysis. We collected two sets of blood to test for CTC detection. For the first set of blood, we used RT-PCR of the *MAGE A1-6* and *hTERT* genes and for the second set of blood we performed CRC culture and analyzed RT-PCR of the *MAGE* and *hTERT* genes in the CRC-cultured CTCs. We defined detection of CTCs as positive expression of the genes by either RT-PCR only or the CRC culture method. The Pearson's Chi-square test was used to compare the detection rate of CTCs using RT-PCR of the *MAGE A1-6* and *hTERT* genes, and the CRC culture method. The association between CTC detection methods and clinicopathologic characteristics was analyzed using Student's t-test for continuous parameters and the Pearson's Chi-square test for categorical data. Analysis of variance (ANOVA; parametric test) or non-parametric Kruskal-Wallis test was performed to compare continuous data of multiple groups. Multiple comparisons tests were performed when there were statistically significant differences between groups. A Bonferroni correction was applied as the post hoc test. All tests were two tailed. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathologic characteristics of the patients. The mean age of the patients was 57.2±12.0 years (mean ± standard deviation; range, 39-82). Among 34 patients, 30 patients had invasive ductal carcinoma, while the others were one each of mucinous carcinoma, metaplastic carcinoma, malignant phyllodes tumor and ductal carcinoma *in situ*. Most of patients with breast cancer were in an early stage, while 1 patient had metastatic breast cancer in the spine. Table I shows patients' clinicopathologic characteristics.

CRC culture of CTCs. CRC culture was carried out on 30 samples except 4 samples lacking sufficient quantity. Following CRC culture, grown CTCs were observed in 7 out of 30 samples (23.3%). Among the samples with growth of CTCs after CRC culture, 4 samples showed positive expression for the *hTERT* and the *MAGE A1-6* genes. The minimum number of cells in the culture plates after CRC culture was 6 cells. Fig. 1 shows the representative CTCs cultured using CRC culture method and the cultured cells were confirmed as cancer cells.

Table I. Patient characteristics.

Clinicopathologic variables	Value
Age (years), mean ± standard deviation (range)	57.2±12.0 (39-82)
Tumor size (cm), mean ± standard deviation (range)	1.8±1.3 (0.4-7.5)
Histologic grade, n (%)	
I	4 (11.8)
II	12 (35.3)
III	18 (52.9)
Lymph node metastasis, n (%)	
Negative	22 (64.7)
Positive	12 (35.3)
Stage, n (%)	
0	1 (2.9)
I	16 (47.1)
II	11 (32.4)
III	5 (14.7)
IV	1 (2.9)
Lymphovascular invasion, n (%)	
Negative	24 (70.6)
Positive	10 (29.4)
ER, n (%)	
Negative	12 (35.3)
Positive	22 (64.7)
PR, n (%)	
Negative	12 (35.3)
Positive	22 (64.7)
HER2 overexpression, n (%)	
Negative	27 (79.4)
Positive	7 (20.6)
Ki-67, n (%)	
<14%	6 (17.6)
≥14%	28 (82.4)
Molecular subtype, n (%)	
Luminal A	5 (14.7)
Luminal B	18 (52.9)
HER2	4 (11.8)
Basal-like	7 (20.6)

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

Comparison of CTC detection rates by methods. The positive expression rates for the *hTERT*, *MAGE A1-6* genes and combination of *hTERT* and *MAGE A1-6* genes in CTCs assessed by RT-PCR only were 15 out of 34 (44.1%), 8 out of 34 (23.5%) and 17 out of 34 (50%), respectively (Figs. 2 and 3). In contrast, CTC detection rates by RT-PCR for the *hTERT*, *MAGE A1-6* genes and combination of *hTERT* and *MAGE A1-6* genes in CTCs grown using CRC culture were 8 out of 30 (26.7%), 3 out of 30 (10.0%) and 10 out of 30 (33.3%), respectively. The

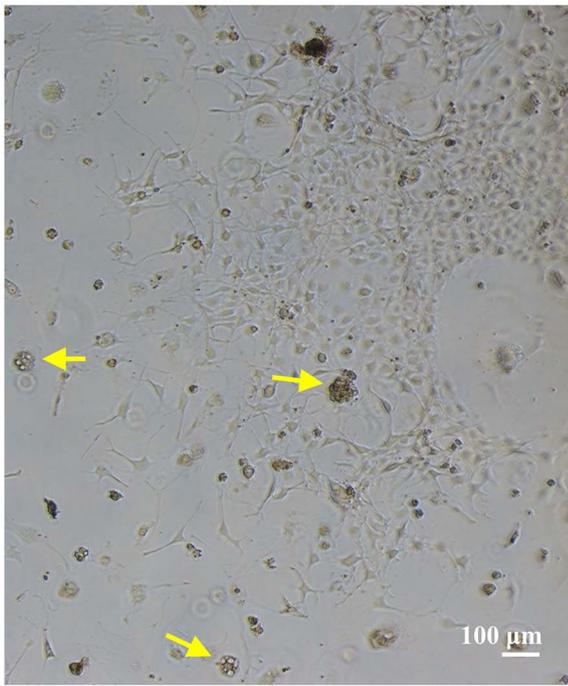


Figure 1. Representative circulating tumor cells (arrows) cultured using the conditionally reprogrammed cell culture method at 4 weeks of culture. Magnification, x40. The cultured cells exhibited positive expression of human telomerase reverse transcriptase as assessed by reverse transcription-PCR and were confirmed to be cancer cells.

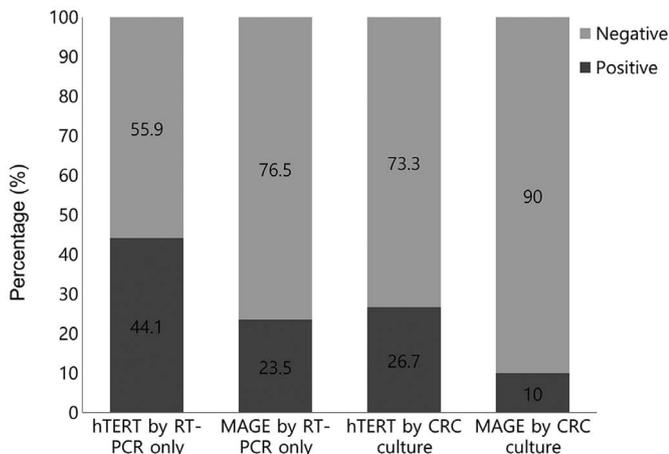


Figure 2. Expression rates of the *hTERT* and the *MAGE A1-6* genes according to the detection methods. RT-PCR only denotes detecting expression of each gene in CTCs by RT-PCR only. CRC culture denotes detecting expression of each gene by RT-PCR in CTCs cultured using the CRC culture method. CRC, conditionally reprogrammed cell; CTCs, circulating tumor cells; *hTERT*, human telomerase reverse transcriptase; *MAGE*, melanoma antigen-encoding gene; RT-PCR, reverse transcription-PCR.

expression of the *GAPDH* gene was 100%. When combining the positive expression rates of RT-PCR only and CRC culture for the *hTERT* and *MAGE A1-6* genes, CTC detection rates increased to 16 out of 30 (53.3%, $P=0.052$) and 7 out of 30 (23.3%, $P=0.304$), respectively. Also, when combining the positive expression rates of both genes by either method, CTC detection rate was 19 out of 30 (63.3%), the highest value observed ($P=0.066$) (Fig. 3). Specifically, in 30 specimens

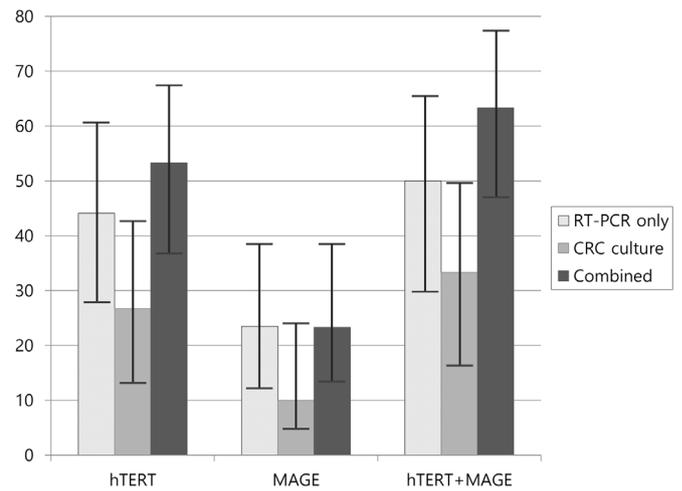


Figure 3. Detection rate of CTCs using the positive expression rates of the *hTERT* and the *MAGE A1-6* genes according to the detection methods. The positive expression rates for the *hTERT* and the *MAGE A1-6* genes in CTCs detected by RT-PCR only were 44.1 and 23.5%, respectively. CTCs detection rates by RT-PCR for the *hTERT* and the *MAGE A1-6* genes in CTCs cultured using CRC culture method were 26.7 and 10.0%, respectively. When combining the positive expression rates of both genes by either method, CTCs detection rate was highest at 63.3% (the right side of the graph). The error bars represented the 95% CI. CRC, conditionally reprogrammed cell; CTCs, circulating tumor cells; *hTERT*, human telomerase reverse transcriptase; *MAGE*, melanoma antigen-encoding gene; RT-PCR, reverse transcription-PCR.

subjected to CRC culture, 5 showed positive expression of the *hTERT* or *MAGE A1-6* gene, although the expression of both genes in the same patients were negative by RT-PCR only.

Association of CTC detection rate with clinicopathologic characteristics by methods. When comparing CTC detection rates between RT-PCR only and CRC culture according to clinicopathologic characteristics of the patients, the positive expression of the *MAGE A1-6* gene by RT-PCR only was significantly associated with higher breast cancer stage and molecular subtype of breast cancer ($P=0.006$ and $P=0.039$, respectively) (Table II). Also, when combining two methods of RT-PCR only and CRC culture, the positive expression rates of the *hTERT* and *MAGE A1-6* genes were significantly higher in stage III or IV breast cancer than in stage I or II breast cancer ($P=0.032$ and $P=0.032$, respectively) (Tables II and III). There was no association between the combined positive expression of both genes and clinicopathologic characteristics (data not shown).

Discussion

Recent advances in research on CTCs have led to the development of multiple technologies to detect and isolate CTCs. In particular, *ex vivo* culture of CTCs using CRC culture methods is thought to be useful for the detection of CTCs and use in CTC-based studies. However, there are few studies on CRC culture in CTC detection. In this study, we aimed to verify the efficacy of CRC culture in CTC detection and as a result, we showed the growth of CTCs after cell culture using CRC culture in breast cancer for the first time. Also, consistent with the results of previous studies (18,19), we found that cultured

Table II. Association between gene expression levels of *MAGE A1-6* of circulating tumor cells and clinicopathological characteristics according to the detection methods in breast cancer.

Clinicopathological variables	RT-PCR only ^a (n=34)		CRC culture ^b (n=30)		Combination ^c (n=33)	
	Positive expression, n (%)	P-value	Positive expression, n (%)	P-value	Positive expression, n (%)	P-value
Tumor size, n (%)						
≤2 cm	4 (17.4)	0.222	1 (4.8)	0.144	5 (22.7)	0.181
>2 cm	4 (36.4)		2 (22.2)		5 (45.5)	
Histologic grade, n (%)						
I	2 (50.0)	0.389	0 (0.0)	0.574	2 (50.0)	0.637
II	2 (16.7)		2 (16.7)		3 (25.0)	
III	4 (22.2)		1 (6.7)		5 (29.4)	
LN metastasis, n (%)						
Negative	3 (13.6)	0.066	3 (14.3)	0.232	5 (23.8)	0.283
Positive	5 (41.7)		0 (0.0)		5 (41.7)	
Stage, n (%)						
I, II	4 (14.3)	0.006 ^d	3 (11.5)	0.474	6 (22.2)	0.032 ^d
III, IV	4 (66.7)		0 (0.0)		4 (66.7)	
LVI, n (%)						
Negative	5 (20.8)	0.666	2 (9.5)	0.894	6 (26.1)	0.444
Positive	3 (30.0)		1 (11.1)		4 (40.0)	
ER, n (%)						
Negative	4 (33.3)	0.320	1 (11.1)	0.894	4 (36.4)	0.592
Positive	4 (18.2)		2 (9.5)		6 (27.3)	
PR, n (%)						
Negative	4 (33.3)	0.320	1 (11.1)	0.894	4 (36.4)	0.592
Positive	4 (18.2)		2 (9.5)		6 (27.3)	
HER2 overexpression, n (%)						
Negative	7 (25.9)	0.518	2 (8.3)	0.543	8 (29.6)	0.858
Positive	1 (14.3)		1 (16.7)		2 (33.3)	
Ki-67, n (%)						
<14%	3 (50.0)	0.092	0 (0.0)	0.414	3 (50.0)	0.246
≥14%	5 (17.9)		3 (12.0)		7 (25.9)	
Molecular subtype, n (%)						
Luminal A	3 (60.0)	0.039 ^d	0 (0.0)	0.715	3 (60.0)	0.240
Luminal B	1 (5.6)		2 (11.1)		3 (16.7)	
HER2	1 (25.0)		0 (0.0)		1 (33.3)	
Basal-like	3 (42.9)		1 (20.0)		3 (42.9)	

^aDetecting expression of *MAGE A1-6* gene in CTCs by RT-PCR only; ^bdetecting expression of the *MAGE A1-6* gene by RT-PCR in CTCs cultured by CRC culture method; ^cdetecting expression of the *MAGE A1-6* gene in CTCs by combining RT-PCR only and CRC culture; ^dStatistically significant (P<0.05). LN, lymph node; LVI, lymphovascular invasion; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; RT-PCR, reverse transcription-quantitative polymerase chain reaction; CRC, conditionally reprogrammed cell.

CTCs preserved the characteristics of the primary cancer cells. Furthermore, our study showed that the detection rate of CTCs was enhanced using a combination of CRC culture methods and RT-PCR of *hTERT* and *MAGE A1-6* genes in the blood.

CRCs have the capacity to grow indefinitely without genetic manipulation (18,22) and CRC culture methods have been

proven to easily establish patient-derived CRC cultures from both normal and cancer tissue (22). In this regard, enhancing the growth of CTCs using CRC culture is helpful in improving the release of viable CTCs and effective in increasing the efficiency of CTC-based studies. In a previous study (19), the authors used a spiking model from metastatic lung cancer

Table III. Association between the gene expression levels of *hTERT* of circulating tumor cells and clinicopathologic characteristics according to the detection methods in breast cancer.

Clinicopathological variables	RT-PCR only ^a (n=34)		CRC culture ^b (n=30)		Combination ^c (n=32)	
	Positive expression, n (%)	P-value	Positive expression, n (%)	P-value	Positive expression, n (%)	P-value
Tumor size, n (%)						
≤2 cm	11 (47.8)	0.529	6 (28.6)	0.719	13 (59.1)	0.631
>2 cm	4 (36.4)		2 (22.2)		5 (50.0)	
Histologic grade, n (%)						
I	3 (75.0)	0.411	2 (66.7)	0.216	4 (100.0)	0.169
II	5 (41.7)		2 (16.7)		6 (50.0)	
III	7 (38.9)		4 (26.7)		8 (50.0)	
LN metastasis, n (%)						
Negative	8 (36.4)	0.218	6 (28.6)	0.719	10 (47.6)	0.174
Positive	7 (58.3)		2 (22.2)		8 (72.7)	
Stage, n (%)						
I, II	11 (39.3)	0.220	6 (23.1)	0.284	13 (48.1)	0.032 ^d
III, IV	4 (66.7)		2 (50.0)		5 (100.0)	
LVI, n (%)						
Negative	11 (45.8)	0.755	6 (28.6)	0.719	13 (59.1)	0.712
Positive	4 (40.0)		2 (22.2)		5 (50.0)	
ER, n (%)						
Negative	4 (33.3)	0.350	3 (33.3)	0.666	5 (50.0)	0.631
Positive	11 (50.0)		5 (23.8)		13 (59.1)	
PR, n (%)						
Negative	5 (41.7)	0.832	3 (33.3)	0.666	6 (33.3)	0.773
Positive	10 (45.5)		5 (23.8)		12 (54.5)	
HER2 overexpression, n (%)						
Negative	12 (44.4)	0.940	7 (29.2)	0.536	15 (57.7)	0.732
Positive	3 (42.9)		1 (16.7)		3 (50.0)	
Ki-67, n (%)						
<14%	4 (66.7)	0.220	0 (0.0)	0.287	4 (66.7)	0.568
≥14%	11 (39.3)		8 (32.0)		14 (53.8)	
Molecular subtype, n (%)						
Luminal A	3 (60.0)	0.775	0 (0.0)	0.575	3 (60.0)	0.817
Luminal B	8 (53.3)		5 (27.8)		10 (55.6)	
HER2	1 (25.0)		1 (33.3)		1 (33.3)	
Basal-like	3 (42.9)		2 (40.0)		4 (66.7)	

^aDetecting expression of the *hTERT* gene in CTCs by RT-PCR only; ^bdetecting expression of the *hTERT* gene by RT-PCR in CTCs cultured using the CRC culture method; ^cdetecting expression of the *hTERT* gene in CTCs by combining RT-PCR only and CRC culture; ^dStatistically significant ($P < 0.05$). LN, lymph node; LVI, lymphovascular invasion; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; RT-PCR, reverse transcription-quantitative polymerase chain reaction; CRC, conditionally reprogrammed cell.

patient and established a CTC line using CRC culture (19). On the other hand, we used CTCs isolated from breast cancer patients, not a spiking model, and *ex vivo* culture of CTCs was possible in breast cancer using CRC culture. Our results suggest that CTC lines can be established by the CRC culture methods using CTCs derived from breast cancer patients, and used for translational research and clinical applications.

Since the first human cancer cell line was established from cervical carcinoma, various cancer cell lines have been generated (14). However, *ex vivo* culture of CTCs has been challenging because of the limited number of viable CTCs. With recent advances of cell culture techniques that optimize culture conditions (14,18), several studies have shown the results of *ex vivo* culture of CTCs in various culture conditions (7,15,17).

Table IV. Comparison between RT-PCR of *hTERT* and *MAGE A1-6* gene and CRC culture: pros and cons.

Pros/cons	RT-PCR of <i>hTERT</i> and <i>MAGE A1-6</i> gene	CRC culture
Pros	Specific for cancer Relatively short test periods	Capacity to grow indefinitely without gene manipulation Easy to establish from patient-derived tissue Cultured cells preserve their original phenotype, potential to distinguish the type of primary cancer Enhancing the growth of viable cells
Cons	False-positive <i>hTERT</i> expression in activated lymphocytes Difficulty in identifying the type of primary cancer Wide range of test sensitivity	Long test periods Requires specific medium and conditions for CRC culture Lack of information on sensitivity and specificity of CRC culture

RT-PCR, reverse transcription-quantitative polymerase chain reaction; CRC, conditionally reprogrammed cell.

Zhang *et al* (15) showed the results of CTC culture from lung cancer using a three-dimensional (3D) co-culture environment using a mix of collagen, matrigel and cancer associated fibroblasts derived from a primary pancreatic tumor as culture medium. Cayrefourcq *et al* (17) established of colon cancer CTC line using stem cell culture medium mixed with DMEM, insulin, EGF, fibroblast growth factor, fetal calf serum and other substances at first, before switching to another culture medium later. In breast cancer, Yu *et al* (7) reported that long-term oligoclonal CTC cultures were established from CTCs isolated from metastatic breast cancer patients. They cultured CTCs in serum-free media supplemented with EGF and basic fibroblast growth factor under hypoxic conditions (4% O₂). Interestingly, the authors tried to test several culture conditions including CRC culture methods, but the results were unsuccessful. On the other hand, unlike previous results (7), our study showed the growth of CTCs using CRC culture in breast cancer. Although the previous study (7) did not explain why the CRC culture methods were unsuccessful in CTC culture, there are several reasons that may explain these conflicting results. First, the technique for isolating CTCs was different from ours and previous studies which used microfluidic technology. Second, we isolated CTCs from patients with primary cancer before surgery, while in the previous study CTCs were isolated from metastatic breast cancer patients who were either off therapy or progressing on treatment. Third, we cultured CTCs using CRC culture method for 4 weeks. In CRC culture methods, epithelial cell colonies are readily visible after 2 days, and cultures usually reach confluence in 5 days (22). For CRC culture of CTCs, culture of CTCs should be started within few hours and once epithelial cell colonies are formed, cultured cells can continuously grow to yield more cells. In previous study, the results showed that 'Fast-growing' tissues such as prostate, lung, cervix, skin and salivary gland tissues can yield up to 2 million cells after 6-7 days (22). Tissues with an intermediate growth rate including breast and kidney tissues yield 1-2 million cells after 2 weeks. 'Slow-growing' tissues such as colon, pancreas, ovary and thyroid tissues yield up to 10,000 cells after 4 weeks. Our CTCs were derived from breast tissue and it was suitable to culture CTCs for at least

2 weeks, so we cultured for 4 weeks to enhance more cell growth. Further clarification requires future studies comparing the various culture conditions.

In addition to the detection of CTCs using CRC culture, we combined RT-PCR of the *hTERT* and *MAGE A1-6* genes to enhance CTC detection rates in breast cancer. The *hTERT* and *MAGE A1-6* genes are known to be specific for cancer. If the positive expression rate of the direct RT-PCR for *hTERT* and *MAGE A1-6* gene is sufficient for the detection of CTCs, CRC culture, which takes 4 weeks, is unnecessary. In previous studies using RT-PCR of the *hTERT* and *MAGE A1-6* genes (13,23), both genes were specifically expressed in CTCs of breast cancer patients. The positive expression levels reported for the *hTERT* and *MAGE A1-6* genes in breast cancer varied from 19.6 to 63.6 and from 13.0 to 63.6%, respectively (13,23-26). Both methods of RT-PCR of *hTERT* and *MAGE A1-6* genes and CRC culture have advantages and disadvantages (Table IV). We hypothesized that if CTC detection using CRC culture is possible, a combination of CRC culture and RT-PCR only for the *hTERT* and *MAGE A1-6* genes in the blood would enhance the detection rate of CTCs. In our results, the detection rate of CTCs using RT-PCR was lower after CRC culture than without which may be due to possible alteration of these genes in cultured CTCs. However, when combining the two methods, CTC detection rates were increased to 53.3 and 23.3% for *hTERT* and *MAGE A1-6* gene, respectively, compared to 26.7 and 10.0% using CRC culture. To the best of our knowledge, this is the first study to show results for the combination of CRC culture and RT-PCR for the *hTERT* and *MAGE A1-6* genes to detect CTCs in breast cancer. Our results suggest that the combination of methods used in this study may be beneficial if the positive expression rate of these genes is not sufficient for the detection of CTCs in breast cancer.

Traditional CTC isolation technologies are based on the removal of normal blood components by chemical lysis of RBCs followed by depletion of CD45 positive leukocytes (9). For CTC capture after this step, a number of technologies have been developed. In our study, we used negative immunomagnetic separation technology using the CD45 antibody capture system (20). Unlike the CellSearch® system which uses positive immunomagnetic

enrichment technology, negative enrichment technologies do not bias the sample according to selection markers (27). Furthermore, while positive immunomagnetic technologies have difficulty in downstream processing due to immobilization of captured CTCs on the surface of the device, negative enrichment can easily retrieve CTCs for further analysis (27). Because the recovery rate and viability of isolated CTCs is important in *ex vivo* culture of CTCs, we preferred negative immunomagnetic separation technology for CRC culture of CTCs. It can also be used in drug susceptibility testing using CTCs.

Although CRC culture of CTCs in breast cancer was shown in this study, the growth rates of CTCs using CRC culture were lower than expected. This may be due to a low number of isolated CTCs, because most of the patients in this study were in an early stage of breast cancer. The detection rate of CTCs in breast cancer is known to be higher in metastatic disease than in early breast cancer (28), and the presence of CTCs has been reported to be associated with disease progression. Our results confirmed these findings, as CTC detection rates were significantly associated with breast cancer stage. Therefore, in early breast cancer, especially stage I, the number of isolated CTCs may be small. In addition, the cell culture conditions can affect the growth of CTCs using CRC culture. In a study to determine optimal growth conditions for CTCs, four different culture environments were tested, and the results showed that a 3D co-culture environment using a mix of collagen, matrigel and cancer associated fibroblasts exhibited the highest level of cell expansion, compared to two-dimensional (2D) or mono culture environments (15). In our study, we used plates containing medium for CRC culture, which is a 2D co-culture environment. Even if CRC culture enables CTCs to grow indefinitely, this 2D environment may have limited the growth of CTCs. To enhance the growth of CTCs in breast cancer, we are planning to test a 3D co-culture environment using matrigel and CRC culture.

Our study has several limitations. First, healthy donor blood was not included so we could not analyze the specificity of the method. We designed this study based on the results of previous studies showing that the *MAGE A1-6* and *hTERT* genes are specifically expressed in cancer cells (29-34). To show more reliable results, a negative control is required. Second, we did not enumerate CTCs after isolation to determine the number of CTCs isolated. Instead of enumeration, we detected CTCs using RT-PCR of the *MAGE A1-6* and *hTERT* genes and it was difficult to determine the average number of isolated CTCs and the percentage of viable isolated CTCs using this method. Therefore, it remains uncertain how many CTCs were culturable after isolation. Further study is required to confirm the proportion of viable CTCs that are considered culturable after isolation. Third, we could not prove that the colonies originated from CTCs. Because we didn't enumerate CTCs after isolation and detected CTCs only using RT-PCR of the *MAGE A1-6* and *hTERT* genes, it was difficult to investigate CTCs microscopically. Instead, we tried to compare the CTC colonies to the microscopic findings of cancer cells in breast cancer tissue. There were some similarities between the CTC colonies and cancer cells in breast cancer tissue, but the findings were not identical. Previous studies described that there is broad morphological and immunophenotypical variation within CTCs derived from the same tumor of origin and detecting CTCs microscopically is still challenged (35-37).

Further investigation is required to prove that the colonies were derived from CTCs. Fourth, we did not evaluate the EpCAM as a positive control for detection of CTCs. Although the detection rate of EpCAM may not be sufficient for detection of CTCs, an old positive control is needed to reliably compare the results. Finally, the blood volume collected in this study was lower than that used in other studies. To increase the sensitivity of our assay methods, it may be necessary to collect more blood. Also, the total number of samples was small, and further studies with a larger sample size and appropriate control group are needed to show more reliable results.

In conclusion, our study shows a potential of CRC culture in the detection of CTCs in breast cancer. We also showed that a combination of CRC culture and RT-PCR for the *hTERT* and *MAGE A1-6* genes is useful in enhancing the detection rate of CTCs in the blood. If we can increase the efficiency of CRC culture to expand CTCs by improving culture conditions, CRC culture could be used for CTC detection in a clinical setting, and CTC lines could be established from breast cancer patients using CRC culture method. To properly apply CRC culture to the utilization of CTCs in breast cancer in clinical applications, further studies with larger numbers of samples and multiple culture conditions are needed.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CHJ, YJJ and SHP developed the study concept. CHJ and YJJ were responsible for the methodology. Formal Analysis, Chang-Ho Jeon performed formal analysis. CHJ and YJJ performed the investigation. SHP and YJJ provided resources. YJJ, SHP and CHJ curated the data. YJJ wrote the original draft. YJJ and CHJ reviewed and edited the manuscript. CHJ was involved in project administration. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval for the study was obtained from the Institutional Review Board of Daegu Catholic University

Hospital. Written informed consent was obtained from all patients according to the protocol.

Patient consent for publication

Written informed consent was obtained from all patients according to the protocol.

Competing interests

The authors declare that they have no competing interests.

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