Melanoma antigen-encoding gene family member A1-6 and hTERT in the detection of circulating tumor cells following CD45- depletion and RNA extraction

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Abstract. A total of 76 blood samples from patients without malignant disease and 107 blood samples from patients with malignant disease were investigated for the presence of circulating tumor cells (CTCs). To detect CTCs, hematopoietic cells were removed from the blood samples and different RNA extraction methods were used to amplify the melanoma antigen-encoding gene family member A1-family member A6 (MAGE A1-6) and the human telomerase reverse transcriptase (hTERT) gene as potential CTC markers. Comparison between four methods for extracting RNA from the blood was performed. The samples were enriched by cluster of differentiation 45 (CD45) antibody capturing, and the reverse transcription-quantitative polymerase chain reaction was used to amplify the MAGE A1-6 and hTERT genes. MAGE A1-6 and hTERT gene expression levels were also evaluated in 14 cancer cell lines, and the MAGE A1-6 and hTERT expression levels were 85.7 and 100%, respectively. The RNeasy method demonstrated the most sensitivity in the SNU1 cells mixed with blood, although the differences between methods was non-significant. The positive expression levels of MAGE A1-6 and hTERT was 11.8% in the control group and 58.9% in those with malignant disease. In the 70 patients with colorectal cancer, positive expression levels of MAGE A1-6 or hTERT were significantly higher in stages T3 and T4 compared with in stages T1 and T2. The CTC detection method involving CD45 antibody capture, RNA extraction and MAGE A1-6 and hTERT reverse transcription resulted in good rates of sensitivity and specificity. Thus, the present study concluded that MAGE A1-6 and hTERT genes may be potential and practical markers for CTCs in a clinical setting.

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Introduction

Although a number of previous studies have attempted to detect circulating tumor cells (CTCs), these have been unable to identify a reliable target so far. The majority of these studies have focused on surface antigens, including the epithelial cell adhesion molecule (1) and cluster of differentiation 45 (CD45) (2). The majority of these methods have involved immunocytochemical detection, but the main drawback of this is the inability to detect cells that do not express these epithelial antigens (3). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) protocols have been developed to enhance sensitivity, but have demonstrated limited specificity (4). If it were possible to target a tumor cell-specific gene, a gene-specific PCR method may prove to be the most sensitive and specific approach to detect CTCs.

The melanoma antigen-encoding gene (MAGE) family are known to be cancer-specific genes, but are poorly expressed in cancer cells (5,6). Common MAGE primers that can detect MAGE family member A1 to MAGE family member A6 (MAGE A1-6) genes simultaneously were developed and have been used in various types of cancer (7-9). The present study investigated the potential of using MAGE A1-6 primers to detect CTCs. The human telomerase reverse transcriptase (hTERT) gene has also been studied in multiple types of cancer cell (10-12). However, because the hTERT gene is expressed in activated lymphocytes (13), it was believed to be of limited use in CTC detection.

The present study used the MAGE A1-6 and hTERT genes individually and in combination to detect CTCs in blood, which had mononuclear cells (MNCs) removed by CD45 antibody capture prior to detection. The removal of MNCs should enhance CTC detection rates (14) and reduce hTERT expression levels in the blood samples. In addition, a number of RNA extraction methods were compared in order to achieve amplification of rare cancer cells in the blood. Finally, the clinical sensitivity and specificity of this CTC detection system was evaluated.

Materials and methods

Cancer cell lines. To determine the expression levels of MAGE and hTERT genes in various cancer cells, 14 cancer cell lines were selected as follows: Five gastric (NCI-N87, SNU1, SNU216, SNU484 and SNU688), four colorectal (CRC1306, SNU1197, SNU1411 and SNU175), one cervical (CaSki), one liver (SK-Hep1), one lung (A549), one breast (MDA-MB-361) and one renal (Caki-1) cancer cell line. All cells were provided by the Korean Cell Line Bank (Seoul, Korea).

Comparison of RNA extraction methods using SNU1 cells. To detect cancer cells that circulate in small numbers in the blood, four commercially available RNA extraction methods were compared: The NucleoSpin kit (Macherey-Nagel GmbH, Düren, Germany), the RNeasy Mini kit (Qiagen AB, Sollentuna, Sweden) and the TRIzol and TRIzol Plus kits (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). SNU1 cancer cells were counted and serially diluted to produce various cell concentrations (5,000, 500, 50 and 5 cells); these solutions were then lysed with the specific cell lysis buffers included with each RNA extraction kit and mixed with 50 ng duck RNA (kindly donated by the Department of Immunology, School of Medicine, Keimyung University, Daegu, Korea), which was used as a carrier RNA to enhance RNA extraction efficiency. The prepared lysis solutions were then subjected to the various RNA extraction methods, according to the manufacturer's protocols, and the MAGE A1-6 and hTERT genes were amplified as subsequently described to compare the RNA extraction efficiency of each reagent.

Patients and blood sample collection. To determine the expression levels of MAGE and hTERT genes in the blood, 76 blood samples (3 ml) from patients without malignant disease were used (age range, 57.8±15.4; male:female ratio, 0.9:1), including 30 samples from healthy people obtained via medical examination, 10 from patients with local inflammation, 9 with trauma, 5 with end-stage renal disease, 5 with central nervous system (CNS) infarction, 4 with heart disease, 3 with CNS hemorrhage, 3 with diabetes, 2 with hepatitis, 2 with pyelonephritis, 2 with gastrointestinal disease and 1 with portal vein thrombosis. Diagnoses of patients with non-malignant diseases were confirmed after physical and medical examinations at the Daegu Catholic University Hospital (Daegu, Republic of Korea) between July 1, 2013 and June 30, 2014.

To detect CTCs, 107 blood samples (3 ml each) were additionally collected from patients with malignant disease (age range, 65.6±12.1; male:female ratio, 1.36:1) between July 1, 2013 and December 30, 2014, including 70 patients with colorectal cancer, 11 with breast cancer, 10 with gastric cancer, 8 with liver cancer, 4 with bile duct cancer, 2 with lung cancer and 2 with pancreatic cancer at the Daegu Catholic University Hospital. Diagnoses of malignant diseases were confirmed following a review of patient medical records and pathology reports. The cancer stage was classified according to the 'T-stage' system by the American Joint Committee on Cancer, 7th edition (15).

Written informed consent was obtained from all patients prior to enrollment in the present study. The study protocols were approved by the Institutional Review Board of the School of Medicine, Catholic University of Daegu.

All blood samples were immediately stored at 4˚C and RNA extraction was performed on the day of sample collection. The blood samples were treated using the CD45 antibody capture system as subsequently described and RNA was extracted from the eluted cells using the RNeasy Mini method, according to the manufacturer's protocol.

Mixing of SNU1 cells with blood and CD45 antibody capture. Blood left over from samples that had been drawn for routine clinical testing from patients with non-malignant disease was used. These remnant blood samples were mixed into one tube, and then divided into 2 ml aliquots. SNU1 cancer cells were counted and serially diluted to produce various cell concentrations (5,000, 500, 50 and 5 cells), and these solutions were then added to the 2 ml aliquots of blood.

To remove the CD45-positive blood cells from the blood samples, the red blood cells were lysed with red blood cell lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany). The MNCs were suspended in 80 μl of CD45 binding buffer and reacted with 20 μl of microbeads conjugated to monoclonal antihuman CD45 antibodies (cat. no., 130-045-801; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 15 min at 4˚C. The $CD45^+$ cells were captured using a magnetic separator, and $CD45^-$ cells were eluted and collected. From the eluted cells, RNA was extracted using the RNeasy Mini kit and TRIzol reagent, and the MAGE A1-6 and hTERT genes were amplified as subsequently described to detect the presence of SNU1 cells in the blood.

Gene amplification of MAGE A1-6, hTERT and GAPDH via RT-qPCR. Extracted RNA from cancer cell lines and blood samples was reverse transcribed using the ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. For reverse transcription, the reaction mixture was incubated at 25˚C for 10 min, 42˚C for 60 min, at 70˚C for 15 min and at 5˚C for 5 min, then stored at -80˚C. The MAGE A1-6, hTERT and GAPDH genes were amplified using the LightCycler FastStart DNA Master System (Roche Diagnostics GmbH) according to the manufacturer's protocol, then detected with a LightCycler 2.0 (Roche Diagnostics GmbH). The MAGE A1-6 gene was amplified with nested PCR. The GAPDH gene was used as a 'housekeeping' gene. Table I contains a list of the primer sequences and thermocycler conditions used.

Statistical analysis. The χ^2 test was used to compare the sensitivity of the four RNA extraction methods and the MAGE A1-6 and hTERT positive expression rates among the patient groups. A paired t-test was used to compare MAGE A1-6 and hTERT expression between disease stage T1/2 and T3/4. Statistical analyses were performed using SPSS version 23.0 software (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

MAGE A1-6, hTERT and GAPDH gene expression levels in cancer cell lines. Analysis of 14 cancer cell lines revealed that the levels of expression of the MAGE A1-6, hTERT and GAPDH genes were 85.7, 100 and 100%, respectively

Table I. Gene-specific primers used for the polymerase chain reaction.

Gene	Sequences	Products (bp)	PCR steps
MAGE A1-6	OF 5'-CTG AAG GAG AAG ATC TGC C-3'	465	95˚C 5 sec, 60˚C 5 sec,
	OR 5'-CCA GCA TTT CTG CCT TTG TGA-3'		72˚C 25 sec
	IF 5'-AAG GAG AAG ATC TGC CAG TG-3'	262	95˚C 5 sec, 62˚C 5 sec,
	IR 5'-GAG GCT CCC TGA GGA CTC T-3'		72˚C 12 sec
hTERT	OF 5-CGG GCT GCT CCT GCG TTT GGT G-3'	311	95˚C 5 sec, 68˚C 5 sec,
	OR 5'-AGC CGC GGT TGA AGG TGA GAC TGG-3'		72˚C 16 sec
GAPDH	OF 5'-TCG GAG TCA ACG GAT TTG GTC GTA-3'	320	95˚C 5 sec, 59˚C 5 sec,
	OR 5'-CAA ATG AGC CCC AGC CTT CTC CA-3'		72˚C 18 sec

MAGE A1-6, melanoma antigen-encoding gene A1-6; hTERT, human telomerase reverse transcriptase; OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse; bp, base pairs.

Table II. Expression levels of MAGE A1-6, hTERT and GAPDH mRNA in the 14 cancer cell lines.

Parameter	MAGE A1-6	hTERT	GAPDH	MAGE Cq	hTERT Cq	GAPDH Cq
Cell line (origin)						
NCI-N87 (gastric)	Neg	Pos	Pos	N/A	26.7	11.0
SNU1 (gastric)	Pos	Pos	Pos	28.8	22.8	19.7
SNU216 (gastric)	Pos	Pos	Pos	28.7	24.5	11.7
SNU484 (gastric)	Pos	Pos	Pos	24.9	21.5	11.8
SNU688 (gastric)	Neg	Pos	Pos	N/A	25.1	11.5
CRC1306 (colorectal)	Pos	Pos	Pos	32.2	26.7	12.6
SNU1197 (colorectal)	Pos	Pos	Pos	32.8	29.6	12.7
SNU1411 (colorectal)	Pos	Pos	Pos	35.1	23.8	12.3
SNU175 (colorectal)	Pos	Pos	Pos	24.7	22	12.1
CaSki (cervical)	Pos	Pos	Pos	31.2	25.7	10.9
SK-Hep1 (liver)	Pos	Pos	Pos	29.6	24.5	16.7
A549 (lung)	Pos	Pos	Pos	29.6	24.5	10.4
MDA-MB-361 (breast)	Pos	Pos	Pos	32.8	29.9	11.0
Caki (renal)	Pos	Pos	Pos	30.9	25.7	10.7
% Positive	85.7	100.0	100.0			
Mean Cq				30.1	25.2	12.5

Neg, negative; Pos, positive; Cq, cycle threshold; MAGE A1-6, melanoma antigen-encoding gene A1-6; hTERT, human telomerase reverse transcriptase.

(Table II). The MAGE A1-6 gene was not expressed in 2 of the 5 gastric cancer cell lines (NCI-N87 and SNU688). The average cycle thresholds of MAGE A1-6, hTERT and GAPDH were 30.1, 25.2 and 12.5, respectively (Table II). In all cell lines, the levels of hTERT expression were higher than MAGE A1-6 expression (Paired t-test, $P<0.001$).

Sensitivity of the four RNA extraction methods for the amplification of MAGE A1-6 and hTERT in SNU1 cells. The positive expression levels of MAGE A1-6 and hTERT in SNU1 cells are summarized in Table III. In the samples containing 5 SNU1 cells, the results obtained using the TRIzol method were deemed the most sensitive, with positive expression levels of 40% for MAGE A1-6 and hTERT genes, although the difference between methods was statistically insignificant. In the samples containing 50 SNU1 cells, the TRIzol and RNeasy Mini methods were the most sensitive tests, with positive expression levels of 60%. On this basis, the TRIzol and RNeasy Mini methods were selected as the RNA extraction methods for the mixed blood samples.

Sensitivity of the four RNA extraction methods for the amplification of MAGE A1-6 and hTERT in blood mixed with SNU1 cells. The positive expression levels of MAGE A1-6 and hTERT in the blood mixed with SNU1 cells are summarized in Table IV. In the samples containing 5 SNU1 cells, the TRIzol and RNeasy Mini methods revealed similar positive expression levels for both genes; although the differences were not

Table III. SNU1 cancer cell detection rates according to RNA extraction method (n=5).

	MAGE A1-6 (%)					hTERT (%)				
No. SNU1 cells	Nucleo Spin	RNeasy Mini	TRIzol	TRIzol Plus	P-value	Nucleo Spin	RNeasy Mini	TRIzol	TRIzol Plus	P-value
5,000	80	100	100	100	0.368	100	100	100	100	1.000
500	60	80	100	100	0.230	60	80	100	100	0.230
50	20	60	60	40	0.528	20	60	80	60	0.279
5	0	0	40	20	0.230	0	0	40	20	0.230

P-values indicate the difference between RNA extraction methods. MAGE A1-6, melanoma antigen-encoding gene; hTERT, human telomerase reverse transcriptase.

Table IV. Cancer cell detection rates in blood mixed with SNU1 cells following cluster of differentiation 45 antibody capturing according to RNA extraction method (n=6).

	MAGE A1-6 (%)			hTERT (%)		
No. SNU1 cells	RNeasy Mini	TRIzol	P-value	RNeasy Mini	TRIzol	P-value
5,000	100.0	100.0	1.000	100.0	100.0	1.000
500	66.7	66.7	1.000	83.3	83.3	1.000
50	50.0	50.0	1.000	50.0	50.0	1.000
5	33.3	16.7	0.605	33.3	50.0	0.334

P-values indicate the difference between RNA extraction methods. MAGE, melanoma antigen-encoding gene A1-6; hTERT, human telomerase reverse transcriptase.

statistically significant, the results obtained using the RNeasy Mini method exhibited consistent positive rates with zero standard deviation. In the samples containing 50 SNU1 cells, the results of the TRIzol and RNeasy Mini methods demonstrated the same positive expression levels. On this basis, the RNeasy Mini method was selected as the final RNA extraction method for CTC detection.

Amplification of MAGE A1-6, hTERT, and GAPDH genes in patients with non-malignant vs. malignant diseases. As presented in Tables V and VI, the positive expression rates of MAGE A1-6, hTERT and MAGE A1-6+hTERT were 5.3, 6.6 and 11.8%, respectively, in the patients with non-malignant diseases and 41.1, 41.1, and 58.9%, respectively, in the patients with malignant diseases. GADPH was amplified in all cases. The hTERT positive expression rates for bile duct, lung and pancreatic cancer were 100%.

MAGE A1-6 or hTERT expression levels are increased in the blood of patients with stage T3 or T4 colorectal cancer. The 70 patients with colorectal cancer were classified by T stage. Though the rates of MAGE A1-6 combined with hTERT expression were not significantly different, the positive expression levels of MAGE A1-6 or hTERT were significantly higher in patients with stage T3 or T4 compared with those in stage T1 or T2 (33.3 vs. 59.2%; P<0.05; Fig. 1).

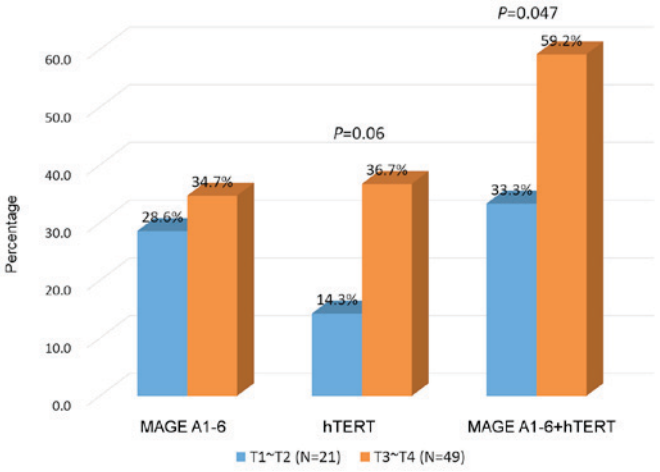

Figure 1. Positive expression levels of MAGE A1-6 and hTERT in the blood of patients with colorectal cancer. MAGE A1-6, melanoma antigen-encoding gene A1-6; hTERT, human telomerase reverse transcriptase.

Discussion

Numerous genetic markers have been used to detect CTCs by RT-qPCR, including cytokeratin 19, carcinoembryonic antigen, hTERT, c-Met and survivin (10,11) and positive expression

Table V. Positive expression levels of GAPDH, MAGE and hTERT in the blood of patients with non-malignant diseases.

	No. positive samples			
Parameter	GAPDH	MAGE A1-6	hTERT	MAGE + hTERT
Diagnosis (n)				
Healthy individuals (30)	30	0	0	0
Local inflammation (10)	10	1	0	1
Trauma (9)	9	0	2	2
End stage renal disease (5)	5	1	0	1
CNS infarction (5)	5	1	0	1
Heart disease (4)	4	1	0	1
CNS hemorrhage (3)	3	0	0	0
Diabetes (3)	3	0	1	1
Hepatitis (2)	2	0	0	0
Pyelonephritis (2)	2	0	1	1
Gastrointestinal diseases (2)	2	0	0	0
Portal vein thrombosis (1)	1	0	1	1
Total number	76	4	5	9
Positive rate (%)	100.0	5.3	6.6	11.8

Age of patients, 57.8±15.4 (mean ± standard deviation); male:female ratio, 0.9:1.0. CNS, central nervous system; MAGE A1-6, melanoma antigen-encoding gene A1-6; hTERT, human telomerase reverse transcriptase.

Table VI. Positive expression levels of GAPDH, MAGE and hTERT in the blood of patients with malignant diseases.

			No. patients with positive expression				
Parameter	T Stage (n)	No. patients	GAPDH	MAGE	hTERT	MAGE + hTERT	Positive rate (%)
Diagnosis							
Colorectal cancer	T1(8), T2(13), T3(35), T4(14)	70	70	23	21	36	51.4
Breast cancer	T1(6), T2(5)	11	11	7	7	8	72.7
Gastric cancer	T1(2), T2(2), T4(6)	10	10	4	4	6	60.0
Liver cancer	T1(2), T2(4), T3(2)	8	8	5	4	5	62.5
Bile duct cancer	T2(1), T3(3)	4	4	1	4	4	100.0
Lung cancer	T3(2)	2	2	2	2	2	100.0
Pancreatic cancer	T3(1), T4(1)	2	2	2	2	2	100.0
Total number		107	107	44	44	63	58.9
Positive rate (%)			100.0	41.1	41.1	58.9	

Age of patients, 65.6±12.1 (mean ± standard deviation); male:female ratio, 1.36:1.00. MAGE A1-6, melanoma antigen-encoding gene A1-6; hTERT, human telomerase reverse transcriptase.

levels varied from 9.6 (CK19, CK20) to 71.2% (MUC1) in patients with gastric cancer, according to the genetic markers used (16,17). microRNA has previously been used to detect tumor cells induced by epithelial-to-mesenchymal transition (18). Pearl *et al* (19) combined antigen and molecular markers to improve CTC detection. A previous study captured tumor cells using epithelial antigens and were able to detect tumor cells using tumor progenitor genes; however, the result was negative rather than positive for the enrichment of tumor cells because CD45 depletion of leukocytes induced significantly greater recovery of spiked hepatocellular carcinoma cells (14).

The present study developed another type of CTC detection system which utilized the known CD45 leukocyte

depletion system along with the novel genetic markers, MAGE A1-6 and hTERT. hTERT mRNA has been used as a genetic marker for CTCs in previous studies (10,11,20); however, the majority of these studies used blood MNCs that contained activated lymphocytes, and these may have induced false-positive hTERT gene expression. In the CD45-depleted cells it was possible to remove the activated lymphocytes, theoretically reducing the rates of false-positive hTERT expression.

The MAGE gene family has previously been studied due to its specific expression in cancer cells (21). Certain studies have used MAGE genes for detecting CTCs (5,6). The present study hypothesized that with CD45-depleted blood cells; a combination of hTERT and MAGE genes would be the most effective markers for CTC detection. MAGE A1-6 and hTERT expression was detected in 12 and 14 cancer cell lines, respectively; only two gastric cancer cell lines (NCI-N87 and SNU688) did not express the MAGE gene. Therefore, it was possible to use these genes as CTC markers.

When comparing RNA extraction efficiency, it was revealed that the TRIzol and RNeasy Mini methods produced the most sensitive results. In a study by Kim *et al* (22), NucleoSpin was identified to be the most effective kit for obtaining high-quality RNA. These authors compared yield and purity of RNA, RNA integrity and cycle threshold values of housekeeping genes, but they did not compare the results of rare gene amplification among normal blood cells (22). In the present study, NucleoSpin failed to amplify the rare target RNA in the blood, whereas the TRIzol and RNeasy Mini methods successfully amplified the MAGE A1-6 and hTERT genes. The TRIzol reagent demonstrated the highest mean RNA yield (22,23) of the RNA extraction kits investigated, with good purity. Furthermore, to amplify rare genes, RNA yields may be the most important factor.

The TRIzol and RNeasy Mini methods revealed similar detection rates for the SNU1 cells in the blood; however, the RNeasy Mini method was more reliable. The fact that the RNA extraction procedure with TRIzol was susceptible to technical variation may explain the variable detection rates for the rare SNU1 cells.

Reported specificities for detection of the hTERT gene in malignant disease have ranged from 67 to 100% (12,20,24,25), but in the present study the specificity was 93%. Healthy individuals and patients with benign disease were included in the control group, whereas previous studies evaluated hTERT specificity based on results in healthy volunteers (12,20,24). Even though the rates of hTERT specificity did not differ greatly between the present study and previous studies, the results of the present study may be efficiently utilized in the clinic.

The sensitivity for the MAGE and hTERT combination in the group with malignant diseases was 58.9%. The expression level of MAGE was lower compared with that of hTERT in the cancer cell lines, but the sensitivities for MAGE were similar to those for hTERT as MAGE expression was amplified using nested PCR. The reported sensitivities of RT-qPCR have varied, including 39 and 70% in breast cancer (21,25), 82% in lung cancer (26), 59% in gastric cancer (27) and 25% in colorectal cancer (28). The majority of studies used 10 ml blood and 3-8 genes as CTC markers, whereas the present study used 3 ml blood and 2 genes. With reduced blood volume and number of genetic markers, the MAGE and hTERT combination should be a more practical approach in the clinical laboratory.

Positive expression levels of the MAGE and hTERT genes in the patients with colorectal cancer are summarized in Fig. 1. Positive expression levels reported using RT-qPCR in patients with colorectal cancer have varied from 25 (28) to 87% (2); the positive expression level of 87% was observed in a study in which blood was obtained from tumor drainage veins from 23 patients, whereas the 25% positive rate was obtained in a study of 735 blood samples from patients with colorectal cancer. Thus, the result of 51.4% revealed in the present study was satisfactory to detect CTCs in the patients with colorectal cancer; however, the number of cases in the present study was not enough to directly compare these results with those in a previous study by Iinuma *et al* (28).

In conclusion, the methods used for blood processing, RNA extraction, and MAGE A1-6 and hTERT reverse transcription resulted in good rates of sensitivity and specificity in the detection of CTCs. The MAGE A1-6 and hTERT genes may serve as markers in this practical approach for CTC detection in the clinical setting.

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