Development and evaluation of a colorimetric membrane-array method for the detection of circulating tumor cells in the peripheral blood of Taiwanese patients with colorectal cancer

JAW-YUAN WANG[1,2], CHING-SHENG YEH[2], YI-FANG CHEN[2], CHAN-HANG WU[2], JAN-SING HSIEH[1], TSUNG-JAN HUANG[1], SUNG-YU HUANG[3] and SHIU-RU LIN[2]

[1]Department of Surgery, Faculty of Medicine, College of Medicine, and Division of Gastrointestinal and General surgery, Department of Surgery, Kaohsiung Medical University Chung-Ho Memorial Hospital, [2]MedicoGenomic Center, Kaohsiung Medical University, Kaohsiung; [3]Sung Hua Biotech Co., Ltd., 11F-1 No. 380 Min-Chiu 2nd Road, Kaohsiung, Taiwan

Received December 7, 2005; Accepted January 23, 2006

Abstract. Early detection is the hallmark of successful cancer treatment. Evidence is accumulating that primary cancers begin shedding neoplastic cells in the circulation at an early stage. To date, a high-sensitivity and high-throughput method for the detection of circulating tumor cells (CTCs) is deficient. In this study, we have developed a high-sensitivity colorimetric membrane-array method to detect CTCs in the peripheral blood of colorectal cancer (CRC) patients as a potential diagnostic tool. Previously, we identified a set of 18 oligonucleotide clones, significantly overexpressed in CRC, which were synthesized and applied to a nylon membrane. Digoxigenin (DIG)-labeled cDNA were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from the peripheral blood of 88 Taiwanese CRC patients and 50 healthy subjects, and were then hybridized to the membrane-array. Hybridization signals were detected by color development. Meanwhile, blood samples were analyzed by real-time quantitative PCR (Q-PCR). Subsequently, both methods were compared regarding their correlation, sensitivity and specificity in the detection of CTCs by statistics. The results of membrane-arrays were demonstrated to be closely related to that of Q-PCR ($P<0.001$). The sensitivity and specificity of membrane-arrays for the detection of CTCs were 94.3% (95% CI, 86.4-102.2%) and 94% (95% CI, 85.9-102.1%), respectively. Moreover, the accuracy of membrane-arrays is higher than that of any one gene by Q-PCR. The detection rate of membrane-arrays was significantly associated with the depth of tumor invasion ($P=0.002$), the presence of lymph node metastasis ($P=0.016$), and TNM stage ($P=0.005$). The preliminary results indicated that the accuracy of membrane-arrays was sufficient to distinguish Taiwanese CRC patients from normal individuals with the advantages of time-saving, cost-effectiveness and high-throughput. Thus, the constructed colorimetric membrane-array could be a promising approach for the future detection of CTCs.

Correspondence to: Dr Shiu-Ru Lin, MedicoGenomic Center, Kaohsiung Medical University, 100 Shin-Chuan 1st Road, Kaohsiung 807, Taiwan
E-mail: srlin@ms2.hinet.net

Key words: membrane-array method, circulating tumor cells, colorectal cancer

Introduction

Colorectal cancer (CRC) is one of the most frequent malignancies and is also the third major cause of cancer-related death in Taiwan, with over 7000 new cases and 3000 deaths per year (http://www.doh.gov.tw/statistic/index.htm; accessed in August 2005). The diagnosis and the therapy of early-stage tumors have the potential to decrease the morbidity and mortality of CRC patients (1). Invasive methods, such as sigmoidoscopy, colonoscopy and double-contrast barium enema, are valuable tools for the specific and sensitive detection and the exact localization of the CRC (2). However, these techniques are unsuitable for broad screening programs mainly because of their low general acceptance and high-invasion (2). Tests for faecal occult blood are non-invasive and useful, especially as an adjunct to sigmoidoscopy. However, the relatively high false-positivity rates and other problems have led to a search for more specific non-invasive tests (3). Since early detection is one of the most effective means of reducing cancer mortality, the development of a sensitive, specific and convenient diagnostic method for detecting CRC at a very early stage is an issue of utmost important.

Evidence is accumulating that primary cancer begins shedding neoplastic cells into the circulation at an early stage (4-7). Thus, circulating tumor cells (CTCs) are a potential source for non-invasive and early diagnosis for CRC patients. Such neoplastic cells may be present in the bloodstream in very low numbers and would be hardly detected by conventional methods. Consequently, a sensitive and powerful method for detection of CTCs is essential, not only to increase the accuracy but also to aid in development of a novel non-invasive diagnostic strategy. Ultimately, such methods will probably redefine

Table I. Primer sequences used for real-time quantitative polymerase chain reaction.

GenBank identity/symbol	Primer	5'-3' sequence	Length
Capping protein (actin filament) muscle Z-line, α 1/CAPZ1	Forward	GTGGTTGGCGTGCTTAAGAT	246
	Reverse	GATTTTGGTGCGGGTAACTG	
Ecotropic viral integration site 2B/EVI2B	Forward	CTTACACCCTGGAAACCAAG	238
	Reverse	CCTGTCCTTCCAAATCCAGA	
ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2/ATP2A2	Forward	GGTGCTCCTGAAGGTGTCAT	241
	Reverse	AGCCAACGAAGGTCAGATTG	
Potassium channel tetramerisation domain containing 2/KCTD2	Forward	TGCAGTTTGTCGAATTGAGG	246
	Reverse	CGCCTGTCTCAAACCTCTTC	
S100 calcium binding protein, ß (neural)/S100B	Forward	CAGGAATTCATGGCCTTTGT	243
	Reverse	CCGTTAAAACAGCCTTTGGA	
Kallikrein 7 (chymotryptic, stratum corneum)/KLK7	Forward	CTCCCAAAGTGCTGGGATTA	250
	Reverse	CCTCATGACGACCCCAGTAT	
Decay accelerating factor for complement (CD55)/DAF	Forward	GGTCCACAGCAGTCGAATTT	246
	Reverse	TGTCAATTTGTGGTGGTGCT	
Guanine nucleotide binding protein, ß polypeptide 2-like 1/G protein	Forward	TGAGTGTGGCCTTCTCCTCT	240
	Reverse	TGTGGTTGGTCTTCAGCTTG	
Embryonic lethal, abnormal vision, Drosophila-like 4 (Hu antigen D)/EVLAVL4	Forward	GAGTCTCTTCGGGAGCATTG	250
	Reverse	CTTCTGGGTCATGGTTTTGG	
Transmembrane 4 superfamily member 3/TM4SF3	Forward	CCAGAGCATATTGCAGGACA	247
	Reverse	TGTCCACAGCAACGTAGGAG	
Kallikrein 1, renal/pancreas/salivary/KLK1	Forward	CTCACAGCTGCTCATTGCAT	245
	Reverse	AACTCCACGACCTTCACAGC	
Formin binding protein 3/FNBP3	Forward	CAGGGCAGCCTTTAACCTTT	248
	Reverse	GTCAGCAGAGACCCAAGAGC	
Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4/SLC25A4	Forward	CCACCCAAGCTCTCAACTTC	240
	Reverse	TGATGATACAGTCGCCCAGA	
H2A histone family, member Y/H2AFY	Forward	GACGGCTTCACAGTCCTCTC	243
	Reverse	AGCAGCTCCAGCTACTTCCA	
WD repeat domain 20/WDR20	Forward	CAACCCCTCTCAAGAGCAAG	243
	Reverse	CCTCTCCTTCCGGTCATGTA	
Nerve growth factor receptor/NGFR	Forward	ATCGGAGGGAATTGAGGTCT	247
	Reverse	AATCCCCACAGGTCACAGTC	
Laminin receptor 1 (67 kD, ribosomal protein SA)/LAMR1	Forward	TCAGTGGGTTTGATGTGGTG	241
	Reverse	CAGACCAGTCTGCAACCTCA	
Chemokine (C-C motif) ligand 20/ CCL20	Forward	CACTCCCAAAGAACTGGGTA	243
	Reverse	GCATTGATGTCACAGCCTTC	
ß-actin	Forward	GCATCCACGAAACTACCTTC	183
	Reverse	CAGGAGGAGCAATGATCTTG	

the most appropriate treatment and affect the outcome for patients with malignancies.

In an attempt to increase the sensitivity of detection of these CTCs, several approaches have been extensively exploited.

In the last decade, the possibility of using molecular detection of CTCs by reverse transcriptase-quantitative polymerase chain reaction (RT-PCR) or real-time quantitative polymerase chain reaction (Q-PCR) of tumor-associated mRNA has received increasing attention. There is evidence that RT-PCR or Q-PCR makes it possible to detect minute quantities of a tumor-related molecular marker in the peripheral blood (8-12). Although these methods provide useful data, one of the limitations was that the methodology could analyze only one molecular target per experiment. A panel of molecular markers would be necessary to increase the sensitivity of CTC detection (13). Applying RT-PCR or Q-PCR for multiple gene expression would be time-wasting and laborious in clinical practice. Therefore, the development of a robust assay for simultaneously detecting CRCs with an excellent high-sensitivity and high-throughput quality, using a panel of informative molecular markers, is imperative. Although peripheral blood is the specimen of choice for a molecular-based technique (14-17), the detection of CTCs in the peripheral blood of CRC patients by a membrane-array method has not yet been reported. The objective of this study was to test our constructed high-sensitivity membrane-arrays that can detect disseminated CRC cells in the peripheral blood. Based on our previous study (18), we selected 18 candidate genes closest related to CRC to develop and construct a colorimetric membrane-array method using the same microarray principle. Subsequently, we also evaluated its diagnostic value in detecting CTCs in the peripheral blood of CRC patients. From our preliminary observation, the membrane-array method is a reliable tool for the detection of CTCs in CRC patients.

Materials and methods

Patients and samples. Eighty-eight patients undergoing elective surgery for CRC at the Department of Surgery of Kaohsiung Medical University Hospital between January 2003 and December 2004 were enrolled in this study. Forty-nine were males and 39 were females. The mean age was 61.9 years (range: 28-84 years). A 4-ml sample of peripheral blood was obtained from each CRC patient during tumor resection or palliative surgery. The same volume of peripheral blood samples taken from 50 healthy individuals served as controls. To prevent contamination of epithelial cells, the peripheral blood samples were obtained through a catheter inserted into a peripheral vessel, and the first 5 ml of blood were discarded. Written informed consent was obtained from all of the subjects and/or guardians for the use of their blood samples. The institutional review board of the Kaohsiung Medical University Hospital approved the sample acquisition and its subsequent use. Clinical stages and pathological features of primary tumors were defined according to the criteria of the American Joint Commission on Cancer (19). Among the 88 CRC patients, there were 11 patients subsequently diagnosed with stage I, 27 patients with stage II, 38 patients with stage III and 12 patients with stage IV classifications.

mRNA isolation and cDNA synthesis. Total RNA was extracted from the fresh whole blood of CRC patients by using a QIAamp® RNA Blood Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically on the basis of absorbance at 260 nm. Poly(A)$^+$-enriched RNA was then purified from total RNA using a Dynabeads® mRNA Direct™ kit (Dynal A.S, Oslo, Norway).

Cell culture. The SW-480 colon cancer cell line was obtained from the American Type Culture Collection, and cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% bovine fetal serum (Gibco-BRL), L-glutamine 2 mmol/l (Sigma-Aldrich, St. Louis, MO) and pyruvate 1 mmol/l (Sigma). Cell cultures were maintained in 5% CO_2 in air, and cells grown in monolayer were harvested by washing the dishes once with phosphate-buffered saline (PBS), pH 7.3, and then incubating the cells with PBS containing EDTA 0.53 mmol/l and 0.05% trypsin (Gibco-BRL) for 10-15 min at 37˚C. Cells were counted and viability was assessed by trypan blue dye exclusion.

Q-PCR. Previously, we had used cDNA microarray and bioinformatics to analyze human genes involved in cell cycle, cell motility, cell adhesion, chemokines, signal transduction, cytoskeleton biogenesis and proteolysis on glass slides, and identified 23 genes related to CRC tumorigenesis and progression (18). In the current investigation, we selected 18 candidate genes closest related to CRC tumorigenesis and progression. The sequences of Q-PCR primers for 18 target genes are listed in Table I. Q-PCR was performed in a Rotor-Gene 2070 thermocycler (Corbett Research Inc., Sydney, Australia). The reaction mixture contained 2 μl of 20 mM dNTP, 2 μl of 30 mM $MgCl_2$, 2 μl of 20X SYBR green, 2 μl of 1 μM primer A, 2 μl of 1 μM primer B, 4 μl of nuclease free water, 2 μl of 80-100 ng/ml cDNA and 2 μl of 1U/μl polymerase. PCR conditions were as follows: 35 cycles of denaturation at 95˚C for 20 sec, annealing at 60˚C for 20 sec, and extension at 74˚C for 40 sec. PCR products were quantified by measuring the fluorescent intensity at the end of each amplification cycle.

Design of oligonucleotide probes and preparation of oligonucleotide membrane-arrays. The procedure for the design and preparation of membrane-array was according to our recent study (20). Using a version of OMP (Oligonucleotide Modeling Platform, DNA Software, Ann Arbor, MN), DNA software tailored to the specifics of membrane-array assay, probe sequences for each target are designed and a single best candidate is selected. Some of the criteria for selection are strong mismatch discrimination, minimal or no secondary structure, the signal strength at the assay temperature, and lack of cross-hybridization. Oligonucleotide probes are synthesized according to well designed sequences, purified and controlled before being grafted onto the substrates. The newly synthesized oligonucleotide fragments were dissolved in di-water to a concentration of 20 mM and then applied to a BioJet Plus 3000 nanoliter dispense system (BioDot Inc., Irvine, CA), which sequentially blotted the 18 target DNAs (Table II), 1 housekeeping gene (ß-actin), and 1 non-mammalian plant gene (50 nl per spot and 1.5 mm between spots) onto a Nytran® SuperCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) and then cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) in triplicate. Each

Table II. Oligonucleotide probe sequences used for membrane-array.

GenBank identity/symbol	Sequence of probe	Length
Capping protein (actin filament) muscle Z-line, α 1/CAPZ1	TGACCACTTACGGAAAGAAGCAAGTGACCCCCAGCCAG AAGAAGCAGATG	50
Ecotropic viral integration site 2B/EVI2B	GCCCCTGCCACCAGTAGATTTTATGAAAAACCAAGAAG ATTCCAACCTTGAGATCCAGTGTC	62
ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2/ATP2A2	CATCGGCATCTTCGGGCAGGATGAGG ACGTGACGTCAAAAGCTTTCACAG	50
Potassium channel tetramerisation domain containing 2/KCTD2	GGAAAGGATACGGGACAATGAGAACAGAACTTCACAA GGCCCCGTGAAGC	50
S100 calcium binding protein, ß (neural)/S100B	CCGAACTCAAGGAGCTCATCAACAATGAGCTTTCCCAT TTCTTAGAGGAAATCAAAGAGCAGGAG	65
Kallikrein 7 (chymotryptic, stratum corneum)/KLK7	TGGAACCACCTGTACTGTCTCCGGCTGGGGCACTACCA CGA	41
Decay accelerating factor for complement (CD55)/DAF	GGGCAGTCAATGGTCAGATATTGAAGAGTTCTGCAATC GTAGCTGCGAGGTG	52
Guanine nucleotide binding protein, ß polypeptide 2-like 1/G protein	ATGACTGAGCAGATGACCCTTCGTGGCACCCTCAAGGG CCACAAC	45
Embryonic lethal, abnormal vision, Drosophila-like 4 (Hu antigen D)/EVLAVL4	GCACCATGGAGCCTCAGGTGTCAAATGGTCCGACATCC AATACAAGCAATG	51
Transmembrane 4 superfamily member 3/TM4SF3	GCAATGACTCTCAAGCAATTTTTGGTTCTGAAGATGTA GGCTCTAGCTCCTACGTTGCTGTG	62
Kallikrein 1, renal/pancreas/salivary/KLK1	GCCTTCTGTCGCCGTCAGAGTGCTGTCTTATGTGAAGTG GATCGAGGACA	50
Formin binding protein 3/FNBP3	CATCATAGGAAACGTTCCCGCTCTCGATCGGGGTCAGA TTCAGATGATGATG	52
Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4/SLC25A4	AGATCTTCAAGTCTGATGGCCTGAGGGGGCTCTACCAG GGTTTCAACGTC	50
H2A histone family, member Y/H2AFY	CACCGAAGCCAGGAAGCCCCGTTTGTAAGCGTGTGTTG TGGTGCTTTATT	50
WD repeat domain 20/WDR20	TAAAGTGGGCTCATTGTCATCCCCAAGCCAGGCCAGTT CTCCAGGTGGAA	50
Nerve growth factor receptor/NGFR	CAAGCGGGAGGAGGTGGAGAAGCTTCTCAACGGCTCT GCG	40
Laminin receptor 1 (67 kD, ribosomal protein SA)/LAMR1	TGCCACTGGAGCCACTCCAATTGCTGGCCGCTTCACTCC TGGAACCTTCA	50
Chemokine (C-C motif) ligand 20/CCL20	GGGCATGGCTATAGCCTTGGCTGTGATATTGTGTGCTA CAGTTGTTCAAGGC	52
ß-actin	TCAGGATTTAAAAACTGGAACGGTGAAGGTGACAGCA GTCGGTTGGAGCGAGCATCCCCC	60

spot consisted of 20 ng of PCR-amplified DNA derived from sequence-verified cDNA clones. DMSO was also dispensed onto the membrane as a blank control (Table III).

Preparation of digoxigenin-labeled cDNA targets and hybridization. First-strand cDNA targets for hybridization were made by reverse transcription of mRNA from the peripheral blood of CRC patients or healthy individuals in the presence of digoxigenin (DIG)-labeled UTP (Roche Diagnostics GmbH, Penzberg, Germany) using SuperScript II reverse transcriptase (Gibco-BRL). The membrane-array needs prehybridization and blocking before hybridization. The lifts were

Table III. Schematic representation of membrane array with 18 genes associated with colorectal cancer, one housekeeping gene, one plant gene, and one blank control.

Plant	CAPZ1	KCTD2	DAF	TM4SF3	SLC25A4	NGFR	ß-actin
Plant	CAPZ1	KCTD2	DAF	TM4SF3	SLC25A4	NGFR	ß-actin
Plant	CAPZ1	KCTD2	DAF	TM4SF3	SLC25A4	NGFR	ß-actin
Blank	EVI2B	S100B	G protein	KLK1	H2AFY	LAMR1	Blank
Blank	EVI2B	S100B	G protein	KLK1	H2AFY	LAMR1	Blank
Blank	EVI2B	S100B	G protein	KLK1	H2AFY	LAMR1	Blank
ß-actin	ATP2A2	KLK7	EVLAVL4	FNBP3	WDR20	CCL20	Plant
ß-actin	ATP2A2	KLK7	EVLAVL4	FNBP3	WDR20	CCL20	Plant
ß-actin	ATP2A2	KLK7	EVLAVL4	FNBP3	WDR20	CCL20	Plant

The symbols represent the genes spotted onto membrane-array, their GenBank identities are listed in Table I.

covered with the ExpressHyb Hybridization Solution (BD Biosciences, Palo Alto, CA) containing DIG-11-UTP-labeled cDNA probes, and then incubated with an anti-digoxigenin alkaline phosphatase conjugated antibody (Roche Diagnostics). We incubated arrays for hybridization at 42˚C for 6 h in a humid chamber. After washing, the array is then exposed to light that excites the light. For signal detection, the membranes were incubated for 15 min in a chromogen solution containing nitroblue-tetrazolium and 5-bromo-4-chloro3-indolyl-phosphate (NBT/BCIP). The hybridized membrane-arrays were then scanned using an Epson Perfection 1670 flat bed scanner (SEIKO EPSON Corp., Nagano-ken, Japan). Subsequent quantification analysis of each spot's intensity was carried out using the commercial software AlphaEase® FC software (Alpha Innotech Corp., San Leandro, CA). Spots consistently differing by a factor of cut-off value calculated from Receiver-operating characteristic (ROC) curves were considered as positive or overexpressed.

Assessment of the diagnostic accuracy of membrane-arrays. Membrane-arrays were used to test a triplicated set of 18 CRC candidate genes for their ability to distinguish the blood of CRC patients from that of controls. The sensitivity, specificity and accuracy of membrane-arrays for the detection of disseminated tumor cells were analyzed. Investigators were blinded to these analyzed blood samples.

Statistical analysis. All data were analyzed using the Statistical Package for the Social Sciences Ver. 10.0 software (SPSS Inc., Chicago, IL). Linear regression and correlation between the Q-PCR and membrane-arrays were analyzed. ROC curves were performed for each target gene in the detection of CTCs by Q-PCR and membrane-arrays to investigate the discrimination accuracy of these two test methods. The area under the ROC curves (AUC) and the corresponding 95% confidence intervals (CI) were calculated for each target gene using both tests. The cut-off with the highest accuracy (minimal false-negative and false-positive results) was determined. Based on the calculated cut-off values, test results were classified as either positive or negative. The sensitivity and specificity of these dichotomous test results and the corresponding 95% CI were determined. Furthermore, we analyzed the accuracy of the membrane-array for distinguishing between the CRC patients and control subjects. ROC curves were constructed by plotting all possible sensitivity/specificity pairs for the membrane-array, resulting from calculating the cut-off values of the number of 18 target genes. The χ^2 test was used to analyze the detection of disseminated tumor cells in peripheral blood by membrane-arrays and the clincopathological features. A probability of <0.05 was considered to be statistically significant.

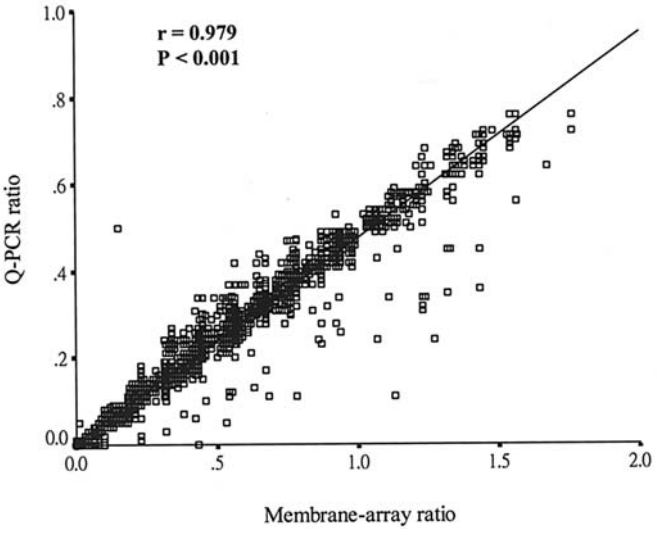

Figure 1. Linear regression and correlation between quantitative PCR (Q-PCR) and membrane-arrays for the detection of 18 target genes in the peripheral blood of 88 colorectal cancer patients and 50 normal individuals.

Results

Each gene expression analysis by Q-PCR and membrane-arrays. The expression of 18 candidate genes in the peripheral blood samples of 88 CRC patients and 50 normal subjects was analyzed simultaneously using Q-PCR and membrane-arrays. Fig. 1 shows the correlation of the gene expression ratio analyzed by Q-PCR and membrane-arrays. There was a highly significant correlation between these two methods (P<0.001, r=0.979). The sensitivity and specificity of each gene by

Table IV. The sensitivity and specificity of each gene by real-time quantitative PCR (Q-PCR) and membrane array for the detection of circulating tumor cells in the peripheral blood of CRC patients and control individuals.

Gene symbol[a]	AUC[b] (95% CI)	Cut-off[c] value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Accuracy[d] (%)
CAPZ1					
Q-PCR	0.904 (0.854-0.954)	0.14	70.5 (60.2-89.8)	92.0 (82.8-100.1)	78.3
Membrane-array	0.921 (0.877-0.964)	0.32	76.1 (61.6-90.7)	90.0 (79.8-100.2)	84.1
EVI2B					
Q-PCR	0.923 (0.876-0.969)	0.34	64.8 (48.5-81.1)	98.0 (93.2-102.8)	75.4
Membrane-array	0.943 (0.905-0.980)	0.59	72.7 (57.5-87.9)	94.0 (85.9-102.1)	80.4
ATP2A2					
Q-PCR	0.885 (0.829-0.941)	0.15	68.1 (52.3-84.1)	88.0 (76.9-99.1)	75.4
Membrane-array	0.903 (0.853-0.952)	0.34	69.3 (53.6-85.1)	90.0 (79.8-100.2)	76.8
KCTD2					
Q-PCR	0.616 (0.516-0.716)	0.11	38.6 (22.0-55.2)	68.0 (52.1-83.9)	49.3
Membrane-array	0.690 (0.594-0.786)	0.26	34.1 (17.9-50.3)	78.0 (63.9-92.1)	50.0
S100B					
Q-PCR	0.829 (0.762-0.896)	0.15	70.5 (54.9-86.0)	82.0 (68.9-95.1)	74.6
Membrane-array	0.866 (0.808-0.924)	0.32	75.0 (60.2-89.8)	82.0 (68.9-95.1)	77.5
KLK7					
Q-PCR	0.847 (0.784-0.910)	0.08	80.7 (67.2-94.2)	76.0 (61.4-90.6)	79.0
Membrane-array	0.868 (0.809-0.927)	0.16	80.7 (67.2-94.2)	80.0 (66.4-93.6)	80.4
DAF					
Q-PCR	0.986 (0.971-1.000)	0.34	88.6 (77.8-99.5)	96.0 (89.3-102.7)	91.3
Membrane-array	0.997 (0.993-1.000)	0.68	85.2 (73.1-97.3)	98.0 (93.2-102.8)	89.9
G protein					
Q-PCR	0.877 (0.821-0.943)	0.15	75.0 (60.2-89.8)	90.0 (79.8-100.2)	80.4
Membrane-array	0.905 (0.856-0.955)	0.32	77.3 (63.0-91.6)	88.0 (76.9-99.1)	81.2
EVLAVL4					
Q-PCR	0.723 (0.631-0.814)	0.11	54.6 (37.6-71.5)	74.0 (59.0-89.0)	61.6
Membrane-array	0.754 (0.668-0.840)	0.23	45.5 (28.5-62.4)	78.0 (63.9-92.1)	57.2
TM4SF3					
Q-PCR	0.840 (0.772-0.908)	0.31	77.3 (63.0-91.6)	80.0 (66.4-93.6)	78.3
Membrane-array	0.847 (0.781-0.912)	0.62	65.9 (49.7-82.1)	86.0 (74.2-97.8)	73.2

Table IV. Continued.

Gene symbol[a]	AUC[b] (95% CI)	Cut-off[c] value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Accuracy[d] (%)
KLK1					
Q-PCR	0.729 (0.645-0.812)	0.11	56.8 (39.9-73.7)	78.0 (63.9-92.1)	64.5
Membrane-array	0.756 (0.678-0.835)	0.24	47.7 (30.7-64.8)	90.0 (79.8-100.2)	63.0
FNBP3					
Q-PCR	0.950 (0.915-0.986)	0.34	80.7 (67.2-94.2)	92.0 (82.8-100.1)	84.8
Membrane-array	0.979 (0.960-0.998)	0.70	80.7 (67.2-94.2)	96.0 (89.3-102.7)	86.2
SLC25A4					
Q-PCR	0.944 (0.907-0.981)	0.36	67.1 (51.0-83.1)	92.0 (82.8-100.1)	76.1
Membrane-array	0.972 (0.946-0.997)	0.69	83.0 (70.1-95.8)	94.0 (85.9-102.1)	87.0
H2AFY					
Q-PCR	0.636 (0.539-0.733)	0.11	51.1 (34.1-68.2)	66.0 (49.8-82.2)	56.5
Membrane-array	0.666 (0.573-0.759)	0.24	44.3 (27.4-61.3)	72.0 (56.7-87.3)	54.3
WDR20					
Q-PCR	0.551 (0.449-0.653)	0.06	70.5 (54.9-86.0)	38.0 (21.4-54.6)	58.7
Membrane-array	0.571 (0.469-0.673)	0.12	73.9 (58.9-88.9)	40.0 (23.3-56.7)	61.6
NGFR					
Q-PCR	0.963 (0.934-0.992)	0.34	86.4 (74.7-98.1)	90.0 (79.8-100.2)	87.7
Membrane-array	0.986 (0.972-1.000)	0.71	89.8 (79.4-100.0)	96.0 (89.3-102.7)	92.0
LAMR1					
Q-PCR	0.703 (0.607-0.799)	0.11	64.8 (48.5-81.1)	70.0 (54.4-85.6)	66.7
Membrane-array	0.719 (0.626-0.811)	0.22	63.6 (47.2-80.0)	70.0 (54.4-85.6)	65.9
CCL20					
Q-PCR	0.947 (0.911-0.983)	0.38	60.2 (43.5-76.9)	94.0 (85.9-102.1)	72.5
Membrane-array	0.967 (0.939-0.995)	0.66	85.2 (73.1-97.3)	94.0 (85.9-102.1)	88.4

[a]The symbols represent the target genes spotted onto membrane array, their GenBank identities are listed in Table II. [b]Area under the ROC curve. [c]The optimal cut-off value for each target gene is calculated by Receiver-operating characteristic (ROC) curve analysis, and the measurement of gene expression is normalized to ß-actin. [d]Accuracy is defined as the proportion of samples correctly classified into CRC (true positives) or control (true negatives) groups.

Q-PCR and membrane-arrays for the detection of CTCs in the peripheral blood of CRC patients and control individuals are listed in Table IV. The sensitivity, specificity, and accuracy of each gene by membrane-arrays are prominently compatible with those by Q-PCR. The diagnostic accuracy of Q-PCR and membrane-arrays varied from 49.3 to 91.3%, as well as

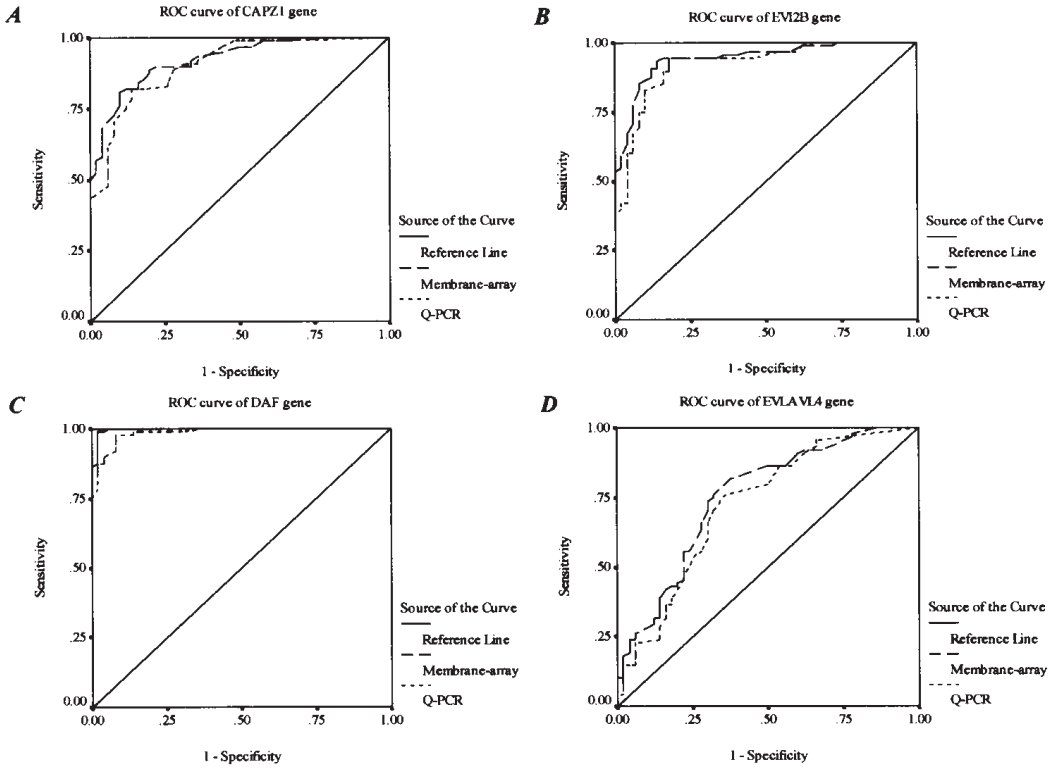

Figure 2. Receiver-operating characteristic (ROC) curves of CAPZ1, EVI2B, DAF and EVLAVL4 genes between quantitative PCR (Q-PCR) and membrane-arrays for the detection of disseminated tumor cells in the peripheral blood of all 138 individuals. The sensitivity on the y-axis was plotted against the false-positive fraction (1-specificity) on the x-axis for various cut-off values. A plot lying above and to the left of another plot indicates greater observed accuracy.

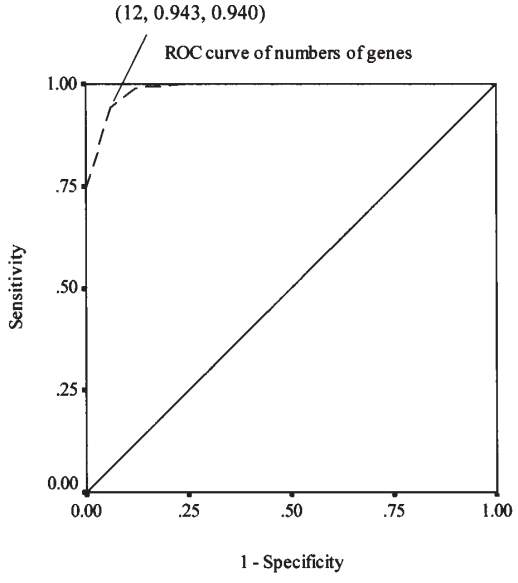

Figure 3. Receiver-operating characteristic (ROC) curves for the membrane-array in the detection of disseminated tumor cells in the peripheral blood samples of all 138 individuals. One of the ROC plots with the number of overexpressed genes of 12 was selected as a cut-off value for the detection of circulating CRC cells. The plot is highlighted with the figures in parenthesis indicating sensitivity and specificity. The area under the ROC curve is 0.988 (95% confidence interval, 0.975-1.000).

from 50.0 to 92%, respectively. The ROC curves in Fig. 2 for 4 selected candidate genes by Q-PCR and membrane-arrays indicate a high accordance between the two test methods.

Diagnostic accuracy of membrane-arrays. ROC curve analyses were performed for the membrane-arrays to detect circulating CRC cells using data from all 138 individuals evaluated. For all 18 target genes, at a cut-off value for the number of 12 positive genes, a sensitivity of 94.3% (95% CI, 86.4-102.2%) and specificity of 94.0% (95% CI, 85.9-102.1%) for membrane-arrays were obtained (Fig. 3). Therefore, a cut-off value of 12 was used for the discrimination between CRC patients and normal individuals. Our preliminary observation showed that there were 3 false positives (6%) and 5 false negatives (5.6%), and a remarkable accuracy of 94.2% and odds ratio of 25.91 (Table V). The detection limitation of membrane-arrays was evaluated using a dilution test. SW-480 colon cancer cells (100, 25, 12, 6 cells) were mixed with 5 ml of peripheral blood obtained from a healthy volunteer. The detection sensitivity of membrane-arrays appears to be better than 25 cancer cells in 5 ml of blood, i.e. 5 cancer cells per 1 ml of blood (Fig. 4). Fig. 5 revealed the hybridization results of membrane-arrays from CRC patients and control individuals. More than 12 genes were overexpressed in the peripheral blood of CRC patients, while ≤ 7 genes were overexpressed in that of normal controls.

Correlation between the detection rate of membrane-arrays and clincopathological features of CRC patients. As shown in Table VI, no statistical significances were observed between the detection rate of membrane-arrays and patients' gender, tumor location, maximum tumor size, or cell differentiation type (all P>0.05). Conversely, the detection rate of membrane-arrays was significantly related to depth of tumor invasion (P=0.002), the presence of lymph node metastasis (P=0.016)

Table V. The detection of disseminated tumor cells in the peripheral blood of CRC patients and control individuals by membrane-arrays.

Membrane-array	CRC patients	Controls	P-value	Odds ratio (95% CI[a])
Positive	83	3	<0.001	25.91 (8.50-79.03)
Negative	5	47		1

[a]CI, confidence intervals; sensitivity, 94.3% (86.4-102.2%); specificity, 94.0% (85.9-102.1%); positive predictive value, 96.5 (90.3-102.8%); negative predictive value, 90.4 (80.3-100.4%); and accuracy, 94.2%.

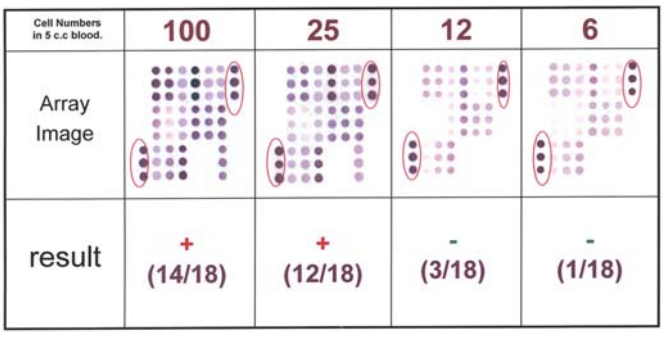

Figure 4. Detection sensitivity of the membrane-array using a diluted CRC cell line. SW-480 cells were mixed with 5 ml of the peripheral blood obtained from a healthy volunteer. According to software analysis, a cell density of 100 cells/5 ml normal blood yielded gene overexpression in 14 of 18 genes and that of 25 cells/5 ml yielded 12 overexpressed genes, both of which were positive for membrane-array analysis. In contrast, a density reduced to 12 cells/5 ml only yielded 3 overexpressed genes, determined to be negative. The genes within the red circles represent ß-actin (positive control).

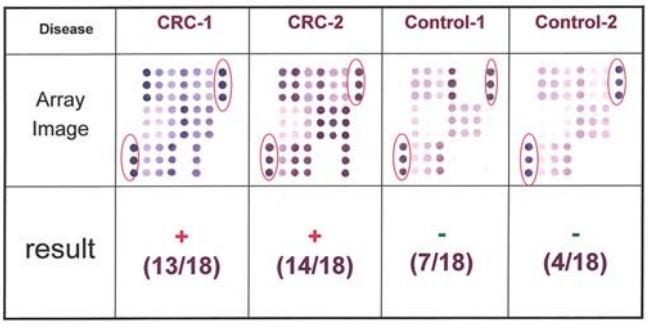

Figure 5. Differential gene expression patterns in a CRC patient and healthy subject. Thirteen or fourteen of a triplicated set of 18 candidate tumor marker genes were overexpressed in the CRC patient, whereas only 7 or 4 genes were overexpressed in the normal control. Thus, the results for the CRC patient and control were interpreted as positive and negative, respectively. The membrane-array consisted of 18 elements of CRC-associated genes; 1 element of the housekeeping gene and 1 plant gene as positive and negative controls were spotted onto the nylon membranes.

and TNM stage (P=0.005). The frequency of stages I, II, III and IV CRC patients detected by membrane-arrays was in 72.7% (8/11), 92.6% (25/27), 100% (38/38) and 100% (12/12), respectively. Moreover, detecting CTCs by membrane-arrays was observed with a tendency of higher detection rate in CRC patients with advanced stage

Table VI. The correlation between the clincopathological features and detection rate of membrane-arrays for circulating tumor cells in colorectal cancer patients.

	Detection rate of membrane-array (%)	P[a]
Total patients	88	
Age (years ± SE)	62.9±1.4	
Sex		0.467
Male	47/49 (95.9)	
Female	36/39 (92.3)	
Location		0.422
Colon	54/58 (93.1)	
Rectum	29/30 (96.7)	
Size		0.801
<5 cm	45/48 (93.8)	
≥ 5 cm	38/40 (95)	
Differentiation		0.055
Well	7/9 (77.8)	
Moderate	57/60 (95)	
Poor	19/19 (100)	
Depth of tumor invasion		0.002
T1	6/9 (66.7)	
T2	12/13 (92.3)	
T3	58/59 (98.3)	
T4	7/7 (100)	
Lymph node metastasis		0.016
No	37/42 (88.1)	
Yes	46/46 (100)	
TNM stage		0.005
I	8/11 (72.7)	
II	25/27 (92.6)	
III	38/38 (100)	
IV	12/12 (100)	

[a]χ^2 test.

Discussion

Using either PCR-based methods (8-12,21,22) or immunocytochemical methods (23), the apparent absence of CTCs in 20-40% of disseminated CRC patients suggests that CTCs were present but were not detected. The main possible reason

contributing to a failure to detect CTCs may be inter-tumoral variation in mRNA expression of the tumor-related genes, because an RT-PCR or Q-PCR assay only selects one or two target molecules. Consequently, a group of candidate genes related to CRC carcinogenesis would probably overcome inter-tumoral variations, and increase the detection rate for CTCs.

Several highly sensitive methods, including cDNA arrays, have been developed to detect CTCs of patients with different types of malignancies (24). Although Takemasa *et al* (25) and Lin *et al* (26) have constructed a cDNA microarray specialized for human CRC development and progression, our present investigation is the first report of membrane-array methods to detect circulating CRC cells to date. The results of this present study showed that a membrane-array method designed from a group of specific oligonucleotide probes for the detection of CTCs in the peripheral blood of CRC patients is a useful, reliable and convenient method. This simple method does not need expensive equipment as the conventional fluorescence based microarray does and, hence, it is economically accessible for the average laboratory. In the present investigation, membrane-arrays were demonstrated to highly significantly correlate (correlation coefficient = 0.979) with Q-PCR for the detection of each target gene expressed in CTCs. Hence, the high-throughput of constructed membrane-arrays would be an advantage when using a set of selected candidate genes for cancer diagnostics. Compared to other published blood-based RT-PCR or Q-PCR assays, whose sensitivity was reported to be between 34 and 88% (8,11,14,15,21,27), this membrane-array assay was found to be more accurate in discriminating CRC patients from normal subjects.

The outstanding 94.2% accuracy and striking correlation to clinical stage of membrane-array assay enables its potential application in the early detection and postoperative surveillance of CRC. Moreover, the detection of CTCs by membrane-arrays was observed with a tendency to a higher detection rate in CRC patients with lymph node metastasis or advanced stage. All T4 tumors, positive lymph mode metastases, stage III and IV patients were identified using the present analysis. Therefore, the identification of CTCs in CRC patients could lead to novel staging approaches, prognostic values and treatment modalities. The membrane-array assay resulted in a false positive rate of 6% (3 out of 50) and a false negative rate of 5.6% (5 out of 88) but it is quite comparable with other immunocytochemical and molecular methods which have been applied to detect occult disseminated tumor cells in CRC patients (8,28-30). In these cases of false positives, it is possible that the membrane-array detected disseminated non-malignant bowel cells which were in the circulation because of inflammation or other normal colorectal processes, and possible contamination of normal skin cells (31). Further testing is necessary to determine the reason for positive molecular marker responses in healthy individuals. In these cases of false negatives, explanations include the possibilty that tumor cells are intermittently flowing into the bloodstream of the bowel and heterogeneity of the tumor cells (23,32,33). Furthermore, image processing or the hybridization process is proposed to be one of the major sources of fluctuations to be expected in array analyses (34). Further improvement of sensitivity may be achieved with usage of immunomagnetic beads for treatment of a large blood volume (32,35), multiple blood sampling (14) or a refined normalization procedure (34). On the other hand, designing new probes to replaces the false positive probes would be helpful in the improvement of the false positive rate and the sensitivity of this membrane-array assay.

Overall, our inexpensive established method for the detection of CTCs in CRC patients seems to be easy and promising, and it contributes to developing a reliable diagnostic tool for CRCs. This novel method uses the same principles as microarray analysis but does not require an expensive arrayer and scanner. Certainly, additional study of a larger patient population by means of a long-term follow-up is mandatory in order to confirm its potential relevance to the detection, prognosis and therapy of CRC.

Acknowledgements

This study was supported by a research grant from the National Science Council of R.O. China (NSC 90-2314-B-037-075).

References

1. Hermanek P: pTNM and residual tumor classifications: problems of assessment and prognostic significance. World J Surg 19: 184-190, 1995.
2. Frazier AL, Colditz GA, Fuchs CS and Kuntz KM: Cost-effectiveness of screening for colorectal cancer in the general population. J Am Med Assoc 284: 1954-1961, 2000.
3. Ahlquist DA and Shuber AP: Stool screening for colorectal cancer: evolution from occult blood to molecular markers. Clin Chim Acta 315: 157-168, 2002.
4. Johnson PW, Burchill SA and Selby PJ: The molecular detection of circulating tumour cells. Br J Cancer 72: 268-276, 1995.
5. Pelkey TJ, Frierson HF Jr and Bruns DE: Molecular and immunological detection of circulating tumor cells and micrometastases from solid tumors. Clin Chem 42: 1369-1381, 1996.
6. Kim SJ, Ikeda N, Shiba E, Takamura Y and Noguchi S: Detection of breast cancer micrometastases in peripheral blood using immunomagnetic separation and immunocytochemistry. Breast Cancer 8: 63-69, 2001.
7. Smirnov DA, Zweitzig DR, Foulk BW, *et al*: Global gene expression profiling of circulating tumor cells. Cancer Res 65: 4993-4997, 2005.
8. Denis MG, Lipart C, Leborgne J, *et al*: Detection of disseminated tumor cells in peripheral blood of colorectal cancer patients. Int J Cancer 74: 540-544, 1997.
9. Ghossein RA, Bhattacharya S and Rosai J: Molecular detection of micrometastases and circulating tumor cells in solid tumors. Clin Cancer Res 5: 1950-1960, 1999.
10. Burchill SA, Lewis IJ and Selby P: Improved methods using the reverse transcriptase polymerase chain reaction to detect tumour cells. Br J Cancer 79: 971-977, 1999.
11. Ito S, Nakanishi H, Hirai T, *et al*: Quantitative detection of CEA expressing free tumor cells in the peripheral blood of colorectal cancer patients during surgery with real-time RT-PCR on a LightCycler. Cancer Lett 183: 195-203, 2002.
12. Vallbohmer D, Zhang W, Gordon M, *et al*: Molecular determinants of cetuximab efficacy. J Clin Oncol 23: 3536-3544, 2005.
13. Racila E, Euhus D, Weiss AJ, *et al*: Detection and characterization of carcinoma cells in the blood. Proc Natl Acad Sci USA 95: 4589-4594, 1998.
14. Wharton RQ, Jonas SK, Glover C, *et al*: Increased detection of circulating tumor cells in the blood of colorectal carcinoma patients using two reverse transcription-PCR assays and multiple blood samples. Clin Cancer Res 5: 4158-4163, 1999.
15. Wong IH, Yeo W, Chan AT and Johnson PJ: Quantitative relationship of the circulating tumor burden assessed by reverse transcription-polymerase chain reaction for cytokeratin 19 mRNA in peripheral blood of colorectal cancer patients with Dukes' stage, serum carcinoembryonic antigen level and tumor progression. Cancer Lett 162: 65-73, 2001.

16. Niiyama H, Mizumoto K, Sato N, *et al*: Quantitative analysis of hTERT mRNA expression in colorectal cancer. Am J Gastroenterol 96: 1895-1900, 2001.
17. Huang P, Wang J, Guo Y and Xie W: Molecular detection of disseminated tumor cells in the peripheral blood in patients with gastrointestinal cancer. J Cancer Res Clin Oncol 129: 192-198, 2003.
18. Wang JY, Yeh CS, Tzou WS, *et al*: Analysis of progressively overexpressed genes in tumorigenesis of human colorectal cancers using cDNA microarray. Oncol Rep 14: 65-72, 2005.
19. Greene FL, Page DL, Fleming ID, *et al*: AJCC cancer staging handbook. Springer-Verlag, New York, pp111-118, 2002.
20. Chen YF, Wang JY, Wu CH, *et al*: Detection of the circulating cancer cells with K-ras oncogene using membrane array. Cancer Lett 229: 115-122, 2005.
21. Jonas S, Windeatt S, O-Boateng A, Fordy C and Allen-Mersh TG: Identification of carcinoembryonic antigen-producing cells circulating in the blood of patients with colorectal carcinoma by reverse transcriptase polymerase chain reaction. Gut 39: 717-721, 1996.
22. Wyld DK, Selby P, Perren TJ, *et al*: Detection of colorectal cancer cells in peripheral blood by reverse-transcriptase polymerase chain reaction for cytokeratin 20. Int J Cancer 79: 288-293, 1998.
23. Molnar B, Ladanyi A, Tanko L, Sreter L and Tulassay Z: Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. Clin Cancer Res 7: 4080-4085, 2001.
24. Martin KJ, Graner E, Li Y, *et al*: High-sensitivity array analysis of gene expression for the early detection of disseminated breast tumor cells in peripheral blood. Proc Natl Acad Sci USA 98: 2646-2651, 2001.
25. Takemasa I, Higuchi H, Yamamoto H, *et al*: Construction of preferential cDNA microarray specialized for human colorectal carcinoma: molecular sketch of colorectal cancer. Biochem Biophys Res Commun 285: 1244-1249, 2001.
26. Lin YM, Furukawa Y, Tsunoda T, Yue CT, Yang KC and Nakamura Y: Molecular diagnosis of colorectal tumors by expression profiles of 50 genes expressed differentially in adenomas and carcinomas. Oncogene 21: 4120-4128, 2002.
27. Fujita S, Kudo N, Akasu T and Moriya Y: Detection of cytokeratin 19 and 20 mRNA in peripheral and mesenteric blood from colorectal cancer patients and their prognosis. Int J Colorectal Dis 16: 141-146, 2001.
28. Soeth E, Vogel I, Roder C, *et al*: Comparative analysis of bone marrow and venous blood isolates from gastrointestinal cancer patients for the detection of disseminated tumor cells using reverse transcription PCR. Cancer Res 57: 3106-3110, 1997.
29. Mori M, Mimori K, Ueo H, *et al*: Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. J Clin Oncol 16: 128-132, 1998.
30. Tsavellas G, Patel H and Allen-Mersh TG: Detection and clinical significance of occult tumour cells in colorectal cancer. Br J Surg 88: 1307-1320, 2001.
31. Smith B, Selby P, Southgate J, Pittman K, Bradley C and Blair GE: Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. Lancet 338: 1227-1229, 1991.
32. Mori M, Mimori K, Ueo H, *et al*: Molecular detection of circulating solid carcinoma cells in the peripheral blood: the concept of early systemic disease. Int J Cancer 68: 739-743, 1996.
33. Liotta LA, Kleinerman J and Saidel GM: Quantitative relationships of intravascular tumor cells, tumor vessels and pulmonary metastases following tumor implantation. Cancer Res 34: 997-1004, 1974.
34. Schuchhardt J, Beule D, Malik A, *et al*: Normalization strategies for cDNA microarrays. Nucleic Acids Res 28: E47, 2000.
35. De Cremoux P, Extra JM, Denis MG, *et al*: Detection of MUC1-expressing mammary carcinoma cells in the peripheral blood of breast cancer patients by real-time polymerase chain reaction. Clin Cancer Res 6: 3117-3122, 2000.