Molecular detection of circulating cancer cells in the peripheral blood of patients with colorectal cancer by using membrane array with a multiple mRNA marker panel

CHING-SHENG YEH[1,2], JAW-YUAN WANG[1,3], CHAN-HAN WU[1], INN-WEN CHONG[4,5], FU-YEN CHUNG[1], YUNG-HSIN WANG[1], YA-PING YU[1] and SHIU-RU LIN[1]

[1]MedicoGenomic Research Center, Kaohsiung Medical University; Departments of [2]Laboratory Medicine, [3]Surgery Medicine, and [4]Internal Medicine, Kaohsiung Medical University Hospital; [5]Kaohsiung Municipal Hsiao-Kang Hospital, Kaohsiung, Taiwan, R.O.C.

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Abstract. The objective of this study was mainly to evaluate the simultaneous detection of expression levels of a multiple mRNA marker panel in the peripheral blood of colorectal cancer (CRC) patients for use in complementary CRC diagnosis. Twenty-seven tumor tissue specimens and 80 peripheral blood specimens were collected from CRC patients. Firstly, the levels of multiple molecular markers in the tumor tissue and blood specimens were evaluated by using real-time quantitative PCR (RT-QPCR) and membrane array. The result of linear regression showed a high degree of correlation (r=0.954, P<0.0001) between the data of these two methods. CK-19 was the marker with the highest detection rate (87.5%) in the peripheral blood, followed by CEA (82.6%), REG4 (80.8%), and then uPA (80.0%) and TLAM1 (80.0%). The levels of the six markers in the peripheral blood were extensively explored. In the 80 patients, the frequency of CK-19, CK-20, CEA, REG4, uPA, and TIAM1 mRNA overexpression was 82.5% (66/80), 78.8% (63/80), 82.5% (66/80), 80.0% (64/80), 78.8% (63/80), and 80.0% (64/80), respectively. Then, a panel combining these 6 mRNA markers was evaluated for its utility in the clinical diagnosis of CRC. The sensitivity, specificity, and accuracy of membrane array-based diagnostic method were 88.8%, 87.8%, and 88.2%, respectively; much higher than those of examinations with single markers. Finally, lymph node metastasis (P=0.024) and TNM stage (P=0.009) were found to be significantly correlated with overexpression of the multiple mRNA marker panel. The detection rates of stage-I and -II CRC by using the multi-marker membrane array were 54.5% (6/11) and 92.0% (23/25), respectively. In conclusion, the results of the present study have shown that this innovative membrane array technique with a multiple mRNA marker panel can significantly improve the diagnosis rate of early colorectal cancer.

Correspondence to: Dr Shiu-Ru Lin, MedicoGenomic Research Center, Kaohsiung Medical University, No. 100, Shin-Chuan 1st Road, Kaohsiung 80317, Taiwan, R.O.C.
E-mail: srlin@ms2.hinet.net

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Introduction

Colorectal cancer (CRC) is a leading cause of morbidity and mortality in Europe and the USA. Each year, approximately 300,000 new cases and 200,000 deaths due to CRC are reported in these areas (1-3). CRC is also one of the most frequent malignancies and the third major cause of cancer-related death in Taiwan, with over 7,000 new cases and 3,000 deaths per year (4). As several investigators have reported, approximately 40-50% of CRC patients who undergo a supposedly curative resection nevertheless subsequently develop metastatic disease, and die of their disease within 5 years (5,6). Survival is closely related to the pathological stage of this disease. There is a dramatic difference in survival rates between patients with early-stage CRC and those with advanced CRC (7,8). Thus, early diagnosis is imperative for obtaining a better therapeutic outcome and prognosis. Although promising advances in imaging technology and other diagnostic modalities have been achieved recently, early detection of CRC remains a challenge. For instance, radiological cross-sectional imaging, such as computed tomography (CT) and magnetic resonance imaging (MRI), meets the required sensitivity (capable of detecting tumor nodules of ≥1 cm in diameter) as a screening tool, but can be ruled out due to high cost; and ultrasonography does not achieve the required sensitivity when used alone (9,10). According to the report of Weidner *et al* (11), active angiogenesis may occur in breast cancer tissue growing to 2 mm in diameter. In a metastatic state, each gram of tumor may shed approximately 10^6 cells into the blood vessel (12). Also, blood sampling is relatively easy. Therefore, disseminated tumor cells in peripheral blood were adopted as a target for early colorectal cancer detection in the present study.

In recent years, several highly sensitive methods have been developed to detect circulating tumor cells in the blood of patients with different types of malignancies, including flow

cytometry (13,14), RT-PCR (15-17), immunohistochemistry (18,19), and Q-PCR (16,20). Despite having been proven to be powerful tools, these techniques are limited to monitor only one or a few markers for each specimen in a single test. In the present study, we exploited membrane array, previously established in our laboratory, as the technological platform for CRC diagnosis (21). This innovative technique could not only monitor multiple markers simultaneously, but also displayed high sensitivity: only 5 cancer cells per 1 ml of blood could be positively detected. Relying on this technological platform, we intended to develop a feasible, highly sensitive, high-throughput CRC diagnosis technique which could screen a panel of informative markers within a short period of time.

The present study was based on the analytic results of TGS-4K array in our previous report (22). By far, a number of mRNA markers including CK-19, CK-20, CEA, REG4, uPA, and TIAM1 have been demonstrated to be up-regulated in CRC cells, and thus widely used for the detection of CRC. Among these markers, CK-19, CK-20, and CEA are the three molecular markers most commonly studied and applied in clinical orientation (23-29). The REG4 gene, recently identified by Dieckgraefe *et al*, plays an import role in the onset of colorectal adenoma, and may be useful for the early diagnosis of CRC (30,31). A large body of clinical data has indicated that high levels of uPA are associated with poor prognosis of CRC patients (32-34). TIAM1 is capable of activating Rac1 as a ubiquitous guanine nucleotide exchange factor and inducing membrane cytoskeleton-mediated cell shape changes, cell adhesion, and cell motility (35,36).

To date, however, the correlation between the expression levels of these mRNA markers and the cancer progression stages of CRC patients requires further elucidation. In the present study, we exploited membrane array to quantitatively analyze the expression of CK-19, CK-20, CEA, REG4, uPA, and TIAM1 mRNA markers in the circulating tumor cells of patients with stage-I and -II (TNM classification) CRC. Hopefully, the goal of early CRC detection can be achieved through this innovative diagnosis technique established in this study and, in turn, improve the efficacy of therapies in managing this malignancy.

Table I. Demographic and clinicopathological characteristics of CRC patients.

	Numbers of patients
Total cases	80
Age (year)	
<60	27
≥60	53
Gender	
Male	42
Female	38
Tumor size[a]	
<2 cm	40
≥2 cm	40
Tumor differentiation	
Well	3
Moderate	64
Poor	13
Depth of tumor invasion	
T1	5
T2	15
T3	57
T4	3
Lymph node metastasis	
Absent	27
Present	53
TNM stage	
I	11
II	25
III	36
IV	8

[a]Tumor size was measured for invasive area by histological examination.

Patients and methods

Patients and samples. Eighty patients undergoing elective surgery for CRC at the Department of Surgery of Kaohsiung Medical University Hospital between April 2003 and May 2005 were enrolled in this study. Forty-two were males and thirty-eight were females. The mean age was 63.24 years (range 27-95 years). Of the 80 patients, 11 were subsequently diagnosed according to the TNM classification system with stage-I, 25 with stage-II, 36 with stage-III, and 8 with stage-IV CRC (Table I). Among 80 pairs of CRC tissue and adjacent normal colorectal tissue surgically removed from the patients, 27 were randomly selected for further analysis, of which 7 presented with stage-I, 7 with stage-II, 7 with stage-III, and 6 with stage-IV disease (data not shown). All surgical tissue samples, upon acquisition, were frozen instantly in liquid nitrogen, and then stored in a -80˚C freezer until analysis. Additionally, a 5-ml sample of peripheral blood was obtained from each of the 80 CRC patients at the time of surgical resection and from 98 healthy volunteers serving as normal controls. To prevent contamination of epithelial cells, 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 subjects and/or guardians for use of their tissue and blood samples. Sample acquisition and subsequent use were also approved by the Institutional Review Board (IRB) of the Kaohsiung Medical University. Clinical stages and pathological features of primary tumors were defined according to the criteria of the American Joint Commission on Cancer (AJCC) (37).

Total RNA extraction and first-strand cDNA synthesis. Total RNA was isolated from patients' blood and tissue specimens

Table II. Primers for real-time Q-PCR and oligonucleotides for membrane arrays.

Primers	Forward primer (5'→3')	Reverse primer (5'→3')
CK-19	ATGAAAGCTGCCTTGGAAGA	TGATTCTGCCGCTCACTATCAG
CK-20	CTGAATAAGGTCTTTGATGACC	ATGCTTGTGTAGGCCATCGA
CEA	AACTGGTGTCCCGGATATCA	ATATTCTTTGCTCCTTGCCA
REG4	CCAAACAGATTTGCAGATCAAGGA	TGCAGGAGTTAGCAGAATCTTGAT
uPA	CTAGGCCTGGGGAAACACAATTACTGCAGG	TGTCTACACGAGGGTCTCACACTTCCTGGA
TIAM1	AAGACGTACTCAGGCCATGTCC	GACCCAAATGTCGCAGTCAG
ß-actin	GCATCCACGAAACTACCTTC	CAGGAGGAGCAATGATCTTG
Oligonucleotides for membrane array (5'→3')		
CK-19	GTATCCGTGTCCTCCGCCCGCTTTGTGTCCTCGTCCTCCT	
CK-20	TCCGCATCTCCAACTCCAGACACACGGTGAACTATGGGAGCGATCTCACA	
CEA	CCGCAGTATTCTTGGCGTATCAATGGGATACCGCAGCAACACACACAAGTTC	
REG4	GAAGCCAGCACCATAGCAGAGTACATAAGTGGCTATCAGAGAAGCCAGCC	
uPA	CTCCAAAGGCAGCAATGAACTTCATCAAGTTCCATCGAACTGTGACTGTCTAAATGGAGGAACATG	
TIAM1	ATGCTGACCGCTTCAAGCTCTACAGTGCCTTCTGCGCCATCCACACAAAA	
ß-actin	TCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTC	

using Isogen™ (Nippon Gene Co., Ltd., Toyama, Japan) and the QiaAmp® RNA blood mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions (38). The RNA concentration was determined spectrophotometrically on the basis of absorbance at 260 nm (Beckman, DU800, USA). First-strand cDNA was synthesized from total RNA by using a RT-PCR kit. Reverse transcription was carried out in a reaction mixture consisting of 1X transcription optimized 5X buffer, 25 μg/ml Oligo(dT) 15 primer, 100 mmol/l PCR nucleotide mix, 200 μmol/l M-MLV reverse transcriptase, and 25 μl of recombinant RNasin® ribonuclease inhibitor (Promega). The reaction mixtures with RNA were incubated at 42˚C for longer than 2 h, heated to 95˚C for 5 min, and then stored at -80˚C until analysis.

Oligo membrane array preparation. We used Visual OMP3 (DNA Software Inc.) to design oligonucleotide probe sequences for target genes and ß-actin served as an internal control (Table II). The newly synthesized oligonucleotide fragments were dissolved in dis-water to a concentration of 20 mM and then applied to a BioJet Plus 3000 nanoliter dispense system, which blotted sequentially the 6 target oligonucleotides and ß-actin (0.05 μl per spot and 1.5 mm between spots) on SuperCharge nylon membrane in triplicate. DMSO was also dispensed onto the membrane as a blank control. After rapid drying and crosslinking procedures, the preparation of colorectal cancer diagnostic membrane array was accomplished (21).

Preparation of digoxigenin-labeled cDNA targets and hybridization. First-strand cDNA targets for hybridization were produced by using SuperScript II reverse transcriptase (Gibco-BRL) in the presence of digoxigenin (DIG)-labeled UTP (Roche Diagnostics GmbH, Penzberg, Germany). After procedures of prehybridization and blocking, the gene chips were subjected to hybridization. The lifts were covered with the Express Hyb Hybridization Solution (BD Biosciences, Palo Alto, CA, USA) containing DIG-11-UTP-labeled cDNA probes, and then incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics). For hybridization, the arrays were incubated at 42˚C for 12 h in a humid chamber. After washing, the arrays were exposed to light. For signal detection, the gene chips were incubated in chromogen solution containing nitroblue-tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP) for 15 min. The hybridize arrays were then scanned with an Epson Perfection 1670 flat bed scanner (Seiko Epson Corp., Nagano-ken, Japan). Subsequent quantification analysis of each spot's intensity was carried out by using AlphaEase® FC software (Alpha Innotech Corp., San Leandro, CA, USA). Spots consistently carrying by a factor of two or more were taken as differentially expressed. These array analysis tools facilitated the measurement of relative gray levels of objects in a uniformly spaced array, such as dot blots. A deformable template extracted the gene spots and quantified their expression levels by the integrated intensity of the spot after background subtraction. The fold ratio for each gene was calculated as follows: spot intensity ratio = (mean intensity of target gene)/(mean intensity of ß-actin).

Cell culture. Human colon adenocarcinoma cell line SW480 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in Leibovitz's L-15 medium (Gibco Life Sciences, BRL, Grand Island, NY) supplemented with 10% of fetal calf serum (FCS) at 37˚C in humidified atmospheric air without CO_2 addition. When grown to confluent monolayer, the cells were harvested by washing the dishes once with phosphate-buffered saline (PBS), pH 7.3, and then incubated in PBS containing 0.53 mmol/l EDTA and 0.05% trypsin (Gibco) for 10-15 min at 37˚C. The trypsinized cells were counted and cell viability was assessed by trypan blue dye exclusion.

A

CK-19	CK-20	CEA	REG4	uPA	TIAM1	Blank	TB	β-actin
CK-19	CK-20	CEA	REG4	uPA	TIAM1	Blank	TB	β-actin
CK-19	CK-20	CEA	REG4	uPA	TIAM1	Blank	TB	β-actin

B

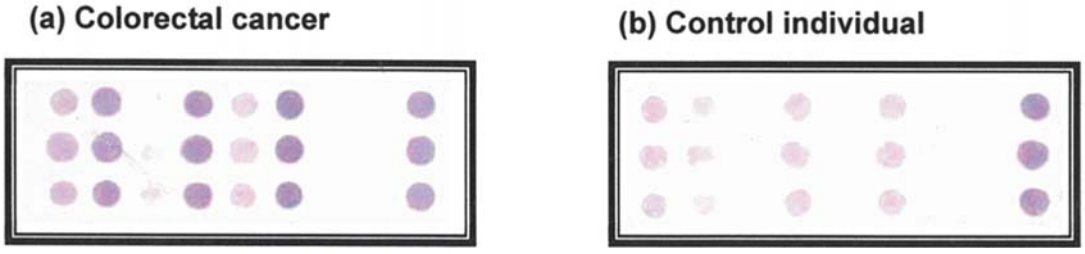

Figure 1. Images of membrane array assay. (A) Positions of oligonucleotide probes on membrane array. CK-19, cytokeratin 19; CK-20, cytokeratin 20; CEA, carcinoembryonic antigen; REG4, regenerating islet-derived family, member 4; uPA, plasminogen activator, urokinase; TIAM1, T-cell lymphoma invasion and metastasis 1; and TB, *Mycobacterium tuberculosis* (negative control). (B) Detection of disseminated cancer cells in peripheral blood by membrane array. (a), CRC patient and (b), normal person.

Sensitivity assay of membrane array. To determine the sensitivity of membrane array assay for CRC cells, SW480 cells of 100, 25, 10, and 5 in number were added to 5 ml of normal peripheral blood. The specimens were subjected to RNA isolation with sequential first-strand cDNA isolation and Dig-dUTP labeling. The Dig-labeled cDNA was then applied to the membrane for hybridization.

Real-time quantitative PCR assay. Real-time Q-PCR was performed in a Rotor-Gene 2070 thermocycler (Corbett Research Inc.). 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 forward primer, 2 μl of 1 μM reverse primer, 4 μl of nuclease free water, 2 μl of 80-100 ng/ml cDNA, and 2 μl of 1 U/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 (i.e., synthesized dsDNA) were quantified by measuring the fluorescent intensity at the end of each amplification cycle. The primers used for real-time Q-PCR are summarized in Table II.

Statistical analysis. All data were analyzed using Statistical Package for the Social Sciences version 11.5 software (SPSS Inc., Chicago, IL). The two-sided χ^2 test and the Fisher exact test were used to compare clinicopathological parameters between mRNA marker-positive patients and mRNA marker-negative patients. Receiver operating characteristics (ROC) curve analyses were carried out to determine the sensitivity and specificity for each mRNA marker (39). The cut-off values for each mRNA marker were set at points representing the highest accuracy of analysis (minimal false-negative and false-positive results). The difference between data obtained by membrane array and real time Q-PCR was calculated by using linear regression and Pearson's correlation. A P-value of less than 0.05 was considered to be statistically significant.

Results

Demography of the study population and correlation between detection data of different methods in mRNA expression levels. Peripheral blood was collected from both 80 CRC patients and 98 healthy volunteers following a standardized procedure. The demographic data and clinicopathological characteristics of all patients are summarized in Table I. The analysis of histological types showed that 3 CRC tissue specimens were identified to be well-differentiated carcinoma, 64 to be moderately-differentiated carcinoma, and 13 to be poorly-differentiated carcinoma. Of the 80 patients, 11 were subsequently diagnosed with stage-I, 25 with stage-II, 36 with stage-III, and 8 with stage-IV CRC. Firstly, total RNA was isolated from blood specimens, and then converted to cDNA, sequentially labeled by Dig-dUTP for membrane hybridization and chromogenesis. Fig. 1 shows the result of membrane array hybridization. Fig. 1A charts the corresponding positions of oligonucleotide probes on membrane array. Fig. 1B(a) and (b) are representative dot patterns of a normal control and a CRC patient, respectively. By comparing the two patterns, it is clear that CK-19, CK-20, CEA, REG4, uPA, and TIAM1 were differentially expressed in CRC. Whether or not there were differences between membrane array data and real-time Q-PCR

Table III. Frequency of mRNA marker overexpression in tumor tissue and peripheral blood of 27 patients with colorectal cancer.

mRNA markers	Overexpression of markers		
	Tissue (%)	Blood (%)	Blood/tissue[a] (%)
CK-19	24 (88.9)	21 (77.8)	21/24 (87.5)
CK-20	24 (88.9)	19 (70.4)	19/24 (79.2)
CEA	26 (96.3)	21 (77.8)	21/26 (82.6)
REG4	26 (96.3)	21 (77.8)	21/26 (80.8)
uPA	25 (92.6)	20 (74.0)	20/25 (80.0)
TIAM1	25 (92.6)	20 (74.1)	20/25 (80.0)

[a]mRNA marker overexpression in the peripheral blood was not found in cases showing no evidence of mRNA marker overexpression in colorectal tumor. The blood-tissue ratio represented the relative detection rate of overexpression for a particular marker in the peripheral blood of colorectal cancer patients showing overexpression of the marker in tumor tissue.

data in levels of CK-19, CK-20, CEA, REG4, uPA, and TIAM1 mRNA expression in the peripheral blood of CRC patients were further statistically analyzed.

Differential expression of mRNA markers in tumor tissue and circulating cancer cells in the peripheral blood of CRC patients. The expression of these molecular markers in the tumor tissue and peripheral blood of CRC patients was detected. In the tumor tissue of 27 CRC patients, the frequency of CK-19, CK-20, CEA, REG4, uPA, and TIAM1 mRNA overexpression was 88.9% (24/27), 88.9% (24/27), 96.3% (26/27), 96.3% (26/27), 92.6% (25/27), and 92.6% (25/27), respectively. Of the patients overexpressing each of these mRNA markers in their tumor tissue, 87.5%, 79.2%, 82.6%, 80.8%, 80.0%, and 80.0% also displayed overexpressed CK-19, CK-20, CEA, REG4, uPA, and TIAM1 mRNA in their peripheral blood, respectively (Table III). This suggested that the 6 mRNA markers had a great potential to be detected in the peripheral blood of CRC patients whose tumor tissue overexpressed the respective genes.

Consistency between data of membrane array and real-time Q-PCR. First, the ROC curves for the six mRNA markers indicated there was a high consistency between the data of membrane array and real-time Q-PCR (Fig. 2). Table IV summarizes the sensitivity and specificity of the two methods for each mRNA marker. The sensitivity and specificity of membrane array for the mRNA markers ranged from 78.8% to 82.5% and from 81.6% to 99.0%, respectively. The sensitivity and specificity of real-time Q-PCR ranged from 70.0% to

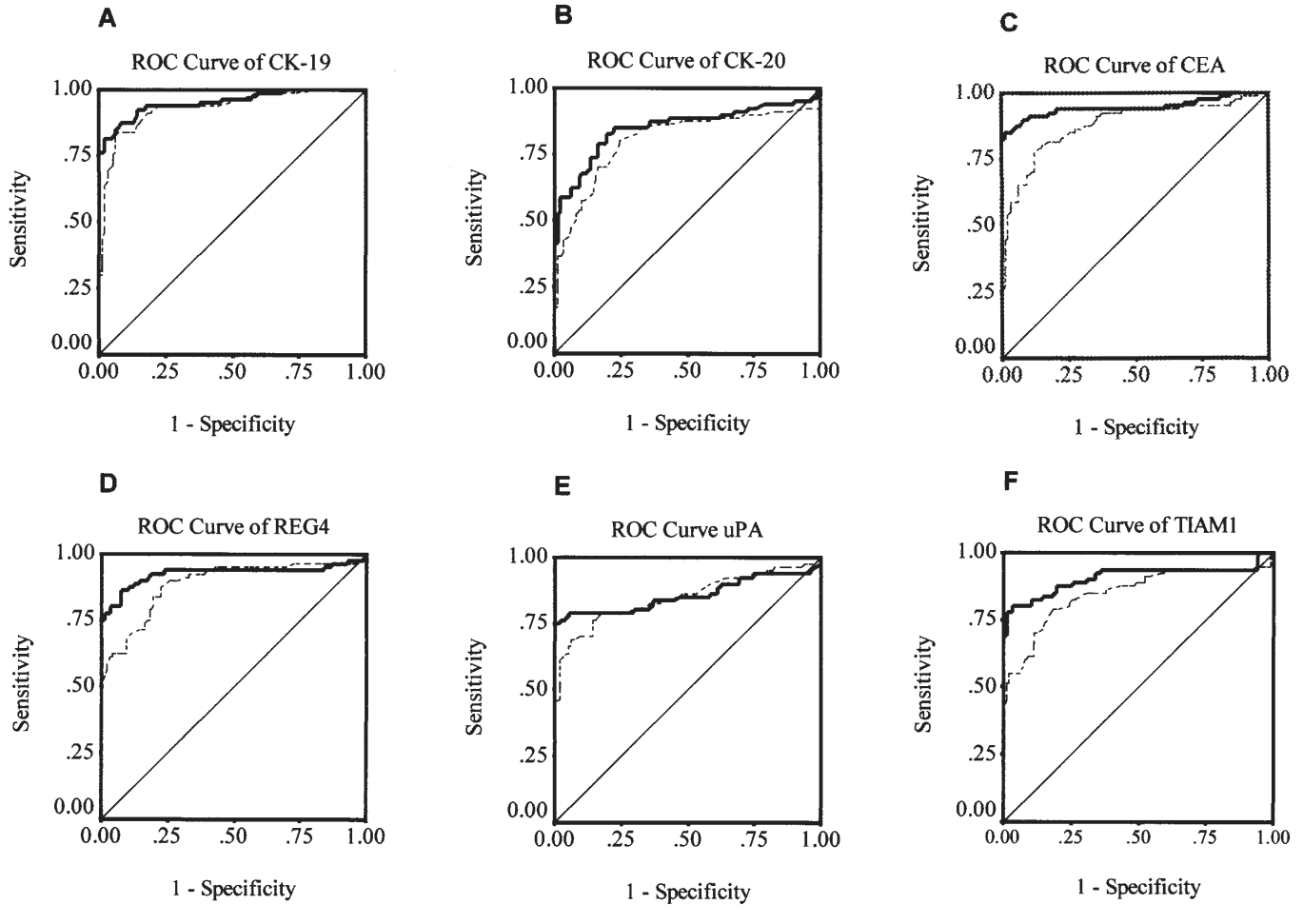

Figure 2. Receiver operating characteristic (ROC) curve analyses for optimum cut-off value, sensitivity, and specificity for mRNA markers: influence of control group. (A-F), CK-19, CK-20, CEA, REG4, uPA, and TIAM1; dashed curve, real-time Q-PCR; solid curve, membrane array.

Table IV. Cut-off value and estimates of sensitivity and specificity for six mRNA markers in peripheral blood of eighty patients measured by two different methods.

Methods	AUC[a]	Cut-off value[b]	Sensitivity (%)	Specificity (%)
CK-19				
Membrane-array	0.925	0.74	82.5	94.9
RT-QPCR	0.932	0.49	78.8	93.9
CK-20				
Membrane-array	0.852	0.53	78.8	81.6
RT-QPCR	0.806	0.33	70.0	80.6
CEA				
Membrane-array	0.944	0.92	82.5	99.0
RT-QPCR	0.879	0.57	77.5	87.8
REG4				
Membrane-array	0.925	0.77	80.0	92.9
RT-QPCR	0.888	0.47	77.5	81.6
uPA				
Membrane-array	0.857	0.86	78.8	93.9
RT-QPCR	0.849	0.53	76.3	85.7
TIAM1				
Membrane-array	0.908	0.73	80.0	89.8
RT-QPCR	0.850	0.44	78.8	80.6

[a]AUC, area under the ROC curve. [b]The cut-off value is a normalized expression value, a ratio of the marker gene expression to the reference gene expression.

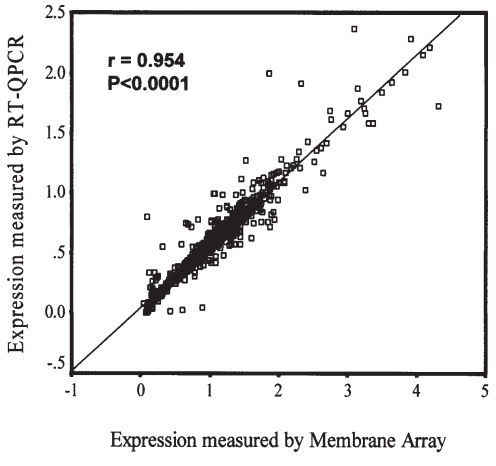

Figure 3. Liner regression and correlation analyses for 480 paired measures of gene expression by real-time Q-PCR and membrane array for six markers in the peripheral blood of 80 patients with colorectal cancer.

78.8% and from 80.6% to 93.9%, respectively (Table IV). The results indicated that both methods had no significant difference in sensitivity and specificity for these markers.

Second, 480 paired measures of expression levels of the six mRNA markers in the 80 CRC patients by the two methods were subjected to linear regression and Pearson's correlation analyses. It demonstrated a high correlation coefficient of r=0.954, which was highly significant (P<0.0001), between the two groups of data (Fig. 3). Therefore, the results of membrane array and real-time Q-PCR assays were highly consistent and correlated with each other. Our membrane array assay showed that the frequency of CK-19, CK-20, CEA, REG4, uPA, and TIAM1 mRNA overexpression in the peripheral blood of CRC patients was 82.5% (66/80), 78.8% (63/80), 82.5% (66/80), 80.0% (64/80), 78.8% (63/80), and 80.0% (64/80), respectively (Table IV).

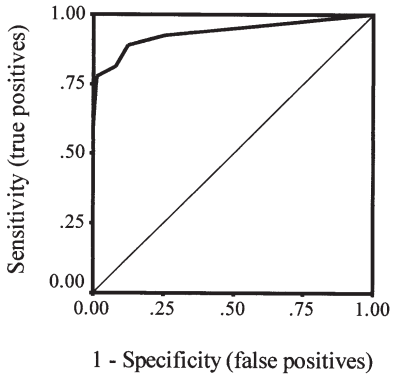

Figure 4. ROC curve analysis of data obtained from membrane array. The outcome of membrane array was defined on the basis of number of markers overexpressed. Sensitivity and specificity of membrane array were determined to be 88.8% and 87.8%, when 3 in the 6-marker panel were overexpressed. Therefore, a membrane array test was defined to be 'positive', when ≥3 markers were overexpressed.

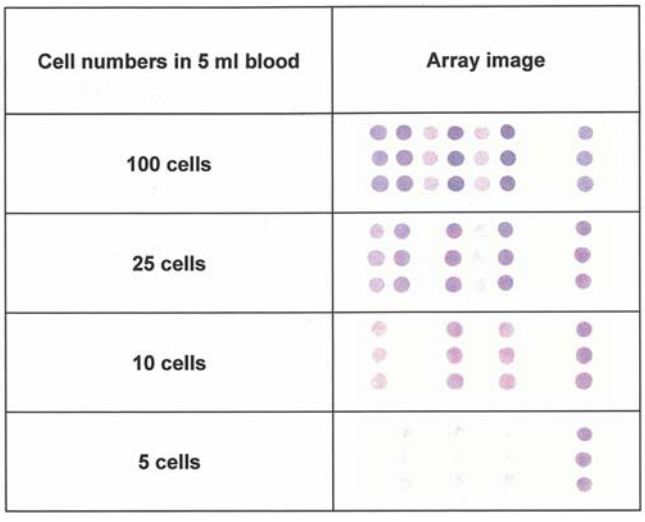

Figure 5. Sensitivity of membrane array. Colorectal cancer cell line SW-480 in numbers of 100, 25, 10, and 5 cells were separately added to 5-ml aliquots of peripheral blood obtained from normal persons. The blood specimens were then subjected to membrane array analysis. The results showed that at least 25 cancer cells in 5 ml of blood could be positively detected. For information on the arrangement of target genes on the membrane array, see Fig. 1A.

Sensitivity, specificity and detection limit of membrane arrays. ROC curve analysis applied to analyze data obtained by membrane array method with a panel combining the 6

Table V. Clinicopathological features of CRC patients with or without mRNA expression in peripheral blood by membrane array.

	CK-19			CK-20			CEA			REG4			uPA			TIAM1		
	+	-	P[b]	+	-	P[b]	+	-	P[b]	+	-	P[b]	+	-	P[b]	+	-	P[b]
No.	66	14		63	17		66	14		64	16		63	17		64	16	
Age (year)			0.864			0.315			0.652			0.156			0.670			0.813
<60	22	5		23	4		23	4		24	3		22	5		22	5	
≥60	44	9		40	13		43	10		40	13		41	12		42	11	
Gender			0.426			0.292			0.166			0.057			0.292			0.823
Male	36	6		35	7		37	5		37	5		35	7		34	8	
Female	30	8		28	10		29	9		27	11		28	10		30	8	
Tumor size[a]			0.556			0.785			0.239			0.576			0.412			1.000
<2 cm	32	8		31	9		31	9		33	7		30	10		32	8	
≥2 cm	34	6		32	8		35	5		31	9		33	7		32	8	
Tumor differentiation			0.120			0.138			0.120			0.660			0.461			0.660
Well	3	0		1	2		3	0		3	0		3	0		3	0	
Moderate	50	14		52	12		50	14		51	13		51	13		51	13	
Poor	13	0		10	3		13	0		10	3		9	4		10	3	
Depth of tumor invasion			0.072			0.303			0.291			0.452			0.522			0.452
T1	4	1		3	2		4	1		4	1		4	1		4	1	
T2	9	6		10	5		10	5		10	5		10	5		10	5	
T3	50	7		48	9		49	8		47	10		46	11		47	10	
T4	3	0		2	1		3	0		3	0		3	0		3	0	
Lymph node metastasis			0.042[c]			0.014[c]			0.008[c]			0.124			0.191			0.124
Absent	19	8		17	10		18	9		19	8		19	8		19	8	
Present	47	6		46	7		48	5		45	8		44	9		45	8	
TNM stage			0.003[c]			0.008[c]			0.042[c]			0.155			0.196			0.155
I	5	6		5	6		6	5		6	5		6	5		6	5	
II	23	2		18	7		22	3		21	4		20	5		21	4	
III	30	6		33	3		30	6		30	6		30	6		30	6	
IV	8	0		7	1		8	0		7	1		7	1		7	1	

[a]Tumor size was measured for invasive area by histological examination. [b]Pearson's χ^2 and Fisher's exact tests were used to compare the differences in proportion of mRNA marker overexpression between different groups of gender, age, tumor size, lymph node metastasis, tumor differentiation, depth of tumor invasion, and TNM stage. [c]P-value for test <0.05.

mRNA markers indicated that the sensitivity, specificity, and accuracy of this method for CRC detection were 88.8%, 87.8%, and 88.2%, respectively, when the optimal cut-off point was 3, i.e. 3 of the 6 mRNA markers were overexpressed in a single diagnostic test (Fig. 4). In order to explore the feasibility and sensitivity of membrane array, SW480 cells of 100, 25, 10, and 5 in number were added to 5-ml aliquots of whole blood obtained from normal persons. CRC cells were positively detected at a level as low as 25 cells/5 ml of blood (Fig. 5). This indicates that membrane array is a feasible and sensitive technique for detecting CRC cells circulating in the bloodstream.

Correlation between the results of membrane array and clinicopathological features of CRC patients. The application of membrane array with 6 mRNA markers using the peripheral blood of an individual in complementary CRC diagnosis was then explored. For single mRNA markers, the overexpression of CK-19, CK-20, and CEA mRNA markers was correlated with lymph node metastasis, and the overexpression of CK-19,

Table VI. Correlation between overexpression of multiple mRNA marker panel in the blood detected by membrane array and clinicopathological characteristics.

	≥3 mRNA markers in overexpression	
Clinicopathological characteristics	Positive case (%)	P-value[a]
TNM stage		0.009[b]
I	54.5 (6/11)	
II	92.0 (23/25)	
III	88.9 (32/36)	
IV	100 (8/8)	
Lymph node metastasis		0.024[b]
Present	92.5 (49/53)	

[a]The comparisons of mean numbers of overexpressed circulating mRNA markers among different pathological stages. [b]P-value for test <0.05.

CK-20, and CEA mRNA was correlated with the TNM stage of CRC (Table V). For the panel of 6 mRNA markers as a whole, a positive detection (≥3 markers over-expressed) was significantly correlated with lymph node metastasis (P=0.024) and TNM stage (P=0.009) of CRC patients (Table VI). In patients with stage-I, -II, -III, and -IV CRC, the detection rates were 54.5% (6/11), 92.0% (23/35), 88.9% (32/36), and 100% (8/8), respectively. In CRC patients with lymph node metastasis, the detection rate was 92.5% (49/53). The over-expression of the other mRNA markers was not significantly correlated with any clinicopathological characteristics of the CRC patients (all P>0.05).

Discussion

The goal of developing the membrane array-based diagnostic method detecting levels of tumor-associated mRNA markers in peripheral blood specimens was to monitor the risk of hematogenous tumor spread and circulating tumor burden in individuals. Our results showed that the membrane array-based method positively detected circulating CRC cells at a density of 5 cells per ml of blood, in agreement with our previous study (21). It was also established that this innovative diagnosis technique achieved a high degree of sensitivity for disseminated tumor cells in the blood, comparable to those of Q-PCR and RT-PCR. Membrane array (40-43), however, is more time-saving and cost-effective than these two PCR-based techniques because it can simultaneously detect multiple target genes. In addition, ROC curve and linear regression analyses demonstrated a high consistency between the data of membrane array and real-time Q-PCR in the expression of 6 mRNA markers in the peripheral blood of CRC patients, indicating that membrane array has great potential for development and promotion in the application to clinical detection of circulating tumor cells in CRC patients. For single markers, the detection rate results obtained by using our membrane array and other methods described in the literature are rather heterogeneous. Some studies found similar low detection rates for certain mRNA markers (44-46). In the present study, the detection rates for particular tumor-associated mRNA markers in the peripheral blood of CRC patients by using membrane array were between 78.8% and 82.5%. The three leading markers with the highest sensitivity were CK-19, CK-20, and CEA mRNA. The prevalence of CEA mRNA expression in the peripheral blood of CRC patients in the literature varies considerably, ranging from 41% to 69% (19,46,47), apparently lower than that in our study. One possible explanation might be related to our blood sampling procedure carried out during the operation. Surgical manipulation is considered to enhance the release of tumor cells into the circulation.

A membrane array-based method simultaneously analyzing 6 mRNA markers could achieve a detection rate for CRC of 88.2% with a sensitivity of 88.8% and specificity of 87.8%. For patients with stage-I and -II CRC, the detection rates of membrane array with multiple mRNA markers was raised to 54.5% and 92.0%, respectively, much higher than the results in the reports of Guadagni *et al* and Hardingham *et al* (45,48). The potential explanations included differences in target genes chosen, designs of oligonucleotide probes (46,49), and detection techniques. Therefore, our findings demonstrated that membrane array simultaneously analyzing the expression of a panel of multiple mRNA markers in the peripheral blood of CRC patients could significantly enhance the detection rate of CRC in the clinical context. In addition, the correlation between the mRNA marker panel and clinicopathological characteristics of patients was explored. As our results showed, overexpression of a majority of mRNA markers in the panel was correlated with lymph node metastasis (P=0.024) and TNM stage (P=0.009) in the CRC patients. This implies that the innovative technological platform for CRC diagnosis can be also used to predict the prognosis of CRC patients. In conclusion, the detection of small numbers of CRC cells in patients' peripheral blood has become more and more important in the diagnosis of this malignancy. The membrane array-based diagnostic method developed in this study can simultaneously monitor levels of CK-19, CK-20, CEA, REG4, uPA, and TIAM1 mRNA markers in an individual's blood with high degrees of sensitivity and feasibility. This method offers a simple, relatively non-invasive, and promising tool for the detection of disseminated tumor cells in CRC patients. So, this innovative diagnosis technique could not only fulfill the demand for early detection of colorectal cancer but also be useful for monitoring the disease's progression. It is hopeful that the application of this membrane array-based method in clinical CRC diagnosis will contribute promising outcomes to post-operative follow-ups and therapeutic treatments for the disease.

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