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Development of a high-throughput membrane-array method for molecular diagnosis of circulating tumor cells in patients with gastric cancers

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Recently several noninvasive methods have been employed to detect circulating tumor cells (CTCs) in cancer patients. In this study, we have developed a highly sensitive, high-throughput colorimetric membrane-array method that was designed to detect a panel of mRNA markers including human telomerase reverse transcriptase (hTERT), cytrokeratin-19 (CK-19), carcinoembryonic antigen (CEA) and mucin 1 (MUC1) mRNA for the presence of CTCs in the peripheral blood of patients with gastric cancer (GC). Digoxigenin-labeled cDNA targets synthesized following total RNA isolation from peripheral blood samples of 64 GC patients and 80 healthy individuals were subjected to membrane-array hybridization. The results showed that membrane array could positively detect 5 cancer cells/ml of peripheral blood in GC cell-dilution experiments. The sensitivity, specificity and diagnostic accuracy for hTERT, CK-19, CEA and MUC1 mRNA ranged from 78.1% to 82.8%, 76.3% to 85% and 81.3% to 83.3%, respectively. Both CEA and MUC1 mRNA expression was correlated significantly with all malignant biological properties of GC, such as macroscopic type, depth of tumor invasion, lymph-node metastasis, TNM stage and coexisting distant metastasis (all $p < 0.05$). Using these 4 markers in combination, the sensitivity, specificity and diagnostic accuracy of membrane array were raised to 89.1%, 91.3% and 90.3%, respectively. The expression of all 4 mRNA markers was an independent predictor for postoperative recurrence/metastasis. GC patients with the expression of all the 4 mRNA markers showed a poorer survival rate than those without the expression of any 1 mRNA marker ($p = 0.0223$). These findings demonstrated that our membrane-array method could detect CTCs in the circulation of GC patients with considerably high sensitivity and specificity. The identification of CTCs in the peripheral blood may be useful in the auxiliary cancer diagnostics or postoperative surveillance of GC patients for recurrence/metastasis.

Key words: circulating tumor cell; membrane array; gastric cancer; molecular diagnosis

The degrees of tumor penetration in the stomach wall and lymph node metastasis have been used for many decades as the 2 major prognostic determinants for gastric cancer (GC) patients. Unfortunately, despite informative staging of patients with GC, some patients with apparently localized disease at diagnosis will subsequently develop recurrent or metastatic diseases.[1–4] One of the major causes is attributed to the presence of disseminated tumor cells shed from the primary carcinoma into circulation, prior to or during surgery. Even in patients with early GC, blood-born metastasis occurs.[2,5,6] Recent attempts to improve staging include sensitive detection of disseminated tumor cells in the blood or lymph nodes by molecular approaches. Evidence increasingly clarifies that primary cancers begin shedding neoplastic cells into the circulation at an early stage.[7–9] It is the dissemination of cancer cells into circulation that is widely accepted as the main cause leading to distant metastasis.[10,11]

By the time a tumor is detected, several molecular changes have already occurred. After decades of basic research attempting to elucidate the underlying molecular mechanisms of carcinogenesis, scientists have discovered a variety of candidate genes with potential usefulness for the early detection of cancer. To date, several tumor-associated mRNA markers have been demonstrated to be absent in normal cells but overexpressed in GC cells, for example, human telomerase reverse transcriptase (hTERT), cytrokeratin-19 (CK-19), carcinoembryonic antigen (CEA) and mucin 1 (MUC1).[12–16] These mRNA markers have been extensively tested for their application to the detection of circulating tumor cells (CTCs) in GC patients.

CTCs are now easily detectable with reverse transcriptase-quantitative polymerase chain reaction (RT-PCR) or real-time quantitative polymerase chain reaction (Q-PCR) of tumor-associated mRNA.[17–20] Although these methods provide valuable information, one of the major limitations is that neither RT-PCR nor Q-PCR is able to analyze more than 1 molecular marker in a single experiment. Several recent RNA-based approaches focus only on clinical significance of single marker analysis. Because of heterogeneity of the expression of tumor-related genes, a multimarker assay is regarded to be more reliable and sensitive than single-marker assay.[20–22] Therefore, a high-throughput assay able to detect simultaneously a panel of informative molecular markers for the presence of CTCs is in urgent need of development, today. The objective of the study was to evaluate the utility of a noninvasive, peripheral blood-based membrane-array assay detecting a panel of tumor-related mRNA markers (hTERT, CK-19, CEA and MUC1) for GC patients. Furthermore, the correlation between the mRNA markers and malignant biological characteristics of GC patients and its potential of application in predicting the prognosis of GC patients after surgery were explored.

Material and methods

Patients and samples

Sixty-four patients undergoing elective surgery for GC at the Department of Surgery of Kaohsiung Medical University Hospital between January 2003 and March 2004 were enrolled in this study. Forty-one were males and 23 were females. The mean age was 60.5 years (range, 36–84). A 4 ml sample of peripheral blood was obtained from each GC patient during the surgical resection of tumor or palliative surgery. Besides, peripheral blood samples were taken from 80 healthy individuals to serve as 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 was discarded. Written informed consent was obtained from all subjects or guardians for the use of

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TABLE I – SEQUENCES OF OLIGONUCLEOTIDE PROBES FOR mRNA MARKERS

mRNA marker	Sequence of probe	Length (bp)
hTERT	AAAGGTGTGCCCTGTACACAGGCGAGGACCCTGCACCTGGAT	42
CK-19	CAACAATTTGTCTGCCTCCAAGGTCCTCTGAGGCAGCAGGCTCTGG	46
CEA	CATGAGAGTCCAGGCTGTCTGAGTCAGCACAGTAAGAAAGTCCTTTCTGCTTTAA	55
MUC1	AATTCCTCTCTGGAAGATCCCAGCACCGACTACTACCAAGAGCTGCAGAG	50
β-actin	TCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTC	55

their blood samples. Sample acquisition and subsequent use were also approved by the institutional review board 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.[23]

mRNA isolation and first strand cDNA synthesis

Total RNA was extracted from the fresh whole blood of GC patients and healthy volunteers by using a QIAamp® RNA Blood Mini Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. The RNA concentration was determined spectrophotometrically on the basis of absorbance at 260 nm.

Cell culture

A GC cell line KATO-III was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% bovine fetal serum (Gibco-BRL), 2 mmol/l L-glutamine (Sigma–Aldrich, St. Louis, MO) and 1 mmol/l pyruvate (Sigma). Cell cultures were maintained at 37°C in an atmosphere of 5% CO_2. After formation of confluent monolayer, cells were washed with phosphate-buffered saline (PBS), pH 7.3, and then incubated with PBS containing 0.53 mmol/l EDTA and 0.05% trypsin (Gibco-BRL) for 10–15 min at 37°C. Cells were counted, and the viability was assessed by trypan blue dye exclusion.

Design of oligonucleotide probes and preparation of oligonucleotide membrane arrays

The procedure for the design and preparation of membrane array was in accordance with our recent work.[24] Visual OMP 3 (Oligonucleotide Modeling Platform, DNA Software, Ann Arbor, MN) was used to design probes for each of the gene targets. The probe selection criteria included strong mismatch discrimination, minimal or no secondary structure, the signal strength at the assay temperature and lack of cross-hybridization. Oligonucleotide probes were then synthesized according to the designed sequences, purified and controlled before being grafted onto the substrates. The newly synthesized oligonucleotide fragments were dissolved in distilled water to a concentration of 20 mM, applied to a BioJet Plus 3000 nanoliter dispense system (BioDot, Irvine, CA), which blotted the 4 target DNAs sequentially (Table I), 1 housekeeping gene (β-actin), and 1 nonmammalian plant gene (50 nl per spot and 1.5 mm between spots) on Nytran® SuperCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate and then cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Each spot contained 20 ng of PCR-amplified DNA derived from sequence-verified cDNA clones. DMSO was also dispensed onto the membrane as a blank control (Table II).

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 GC patients or healthy individuals in the presence of digoxigenin (DIG)-labeled UTP (Roche Diagnostics, Penzberg, Germany), using SuperScript II reverse transcriptase (Gibco-BRL). The membranes were prehybridized and blocked before hybridization. The lifts were covered with the ExpressHyb Hybridization Solution (BD biosciences, Palo Alto, CA) containing DIG-11-UTP-labeled cDNA probes, and then, incubated with anti-digoxigenin alkaline phosphatase conjugated antibody (Roche Diagnostics). Then, the arrays were incubated for hybridization at 42°C for 6 hr in a humid chamber. After washing, the arrays were subjected to light-excited emission. 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, Nagano-ken, Japan). Subsequent quantification analysis of each spot's intensity was carried out using AlphaEase®FC software (Alpha Innotech, San Leandro, CA). Spots consistently displaying signal intensity higher than the cut-off value calculated from receiver-operating characteristic (ROC) curves by at least 1 factor were considered to be positive or overexpressed.

TABLE II – SCHEMATIC REPRESENTATION OF MEMBRANE ARRAY WITH 4 TARGET GENES, 1 HOUSEKEEPING GENE (β-ACTIN), 1 PLANT GENE (NEGATIVE PLANT) AND 1 BLANK CONTROL

β-actin	Negative	hTERT	CK-19	Blank	β-actin
β-actin	Negative	hTERT	CK-19	Blank	β-actin
β-actin	Negative	hTERT	CK-19	Blank	β-actin
Blank	β-actin	CEA	MUC1	β-actin	Negative
Blank	β-actin	CEA	MUC1	β-actin	Negative
Blank	β-actin	CEA	MUC1	β-actin	Negative

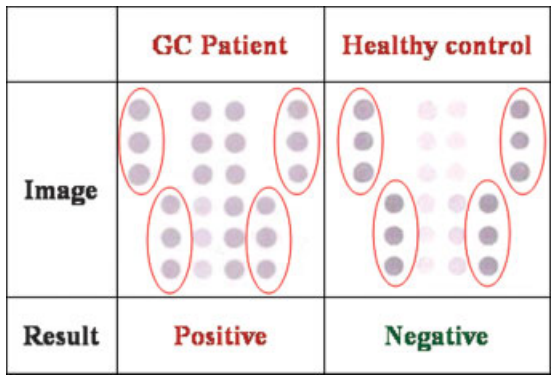

FIGURE 1 – Comparison of gene expression patterns between a GC patient and a healthy control. A triplicated set of 4 molecular markers for GC was blotted on nylon membrane. In addition, a house-keeping gene and a plant gene serving as positive and negative controls were also blotted on the membrane. The spots within the red circle of each image represent β-actin (positive control).

Assessment of the diagnostic accuracy of membrane arrays

The presence of CTCs in the peripheral blood of GC patients and normal controls were detected with membrane arrays on which probes for hTERT, CK-19, CEA and MUC1 genes were blotted in triplicate. Then, the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of membrane array for GC were analyzed. Investigators were blinded to the groups of blood samples.

Detection sensitivity of membrane-array assay

The detection sensitivity of membrane-array assay was evaluated by a cell-dilution study. Cells (100, 50, 25 and 12 cells in number) of a GC cell line KATO-III were mixed with 5 ml aliquots of peripheral blood obtained from a healthy volunteer.

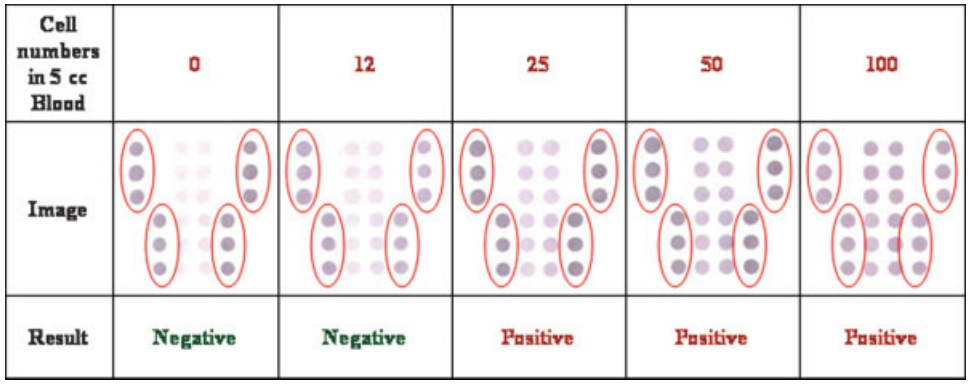

FIGURE 2 – Cell-dilution experiments with GC cell line KATO-III for the detection of sensitivity of membrane array. KATO-III cells at various cell numbers were added in 5 ml of peripheral blood obtained from a healthy volunteer. Signals of mRNA markers remained detectable in 5 ml blood sample containing 25 KATO-III cells, i.e., 5 cells/ml of blood. The spots within the red circle of each image represent β-actin (positive control).

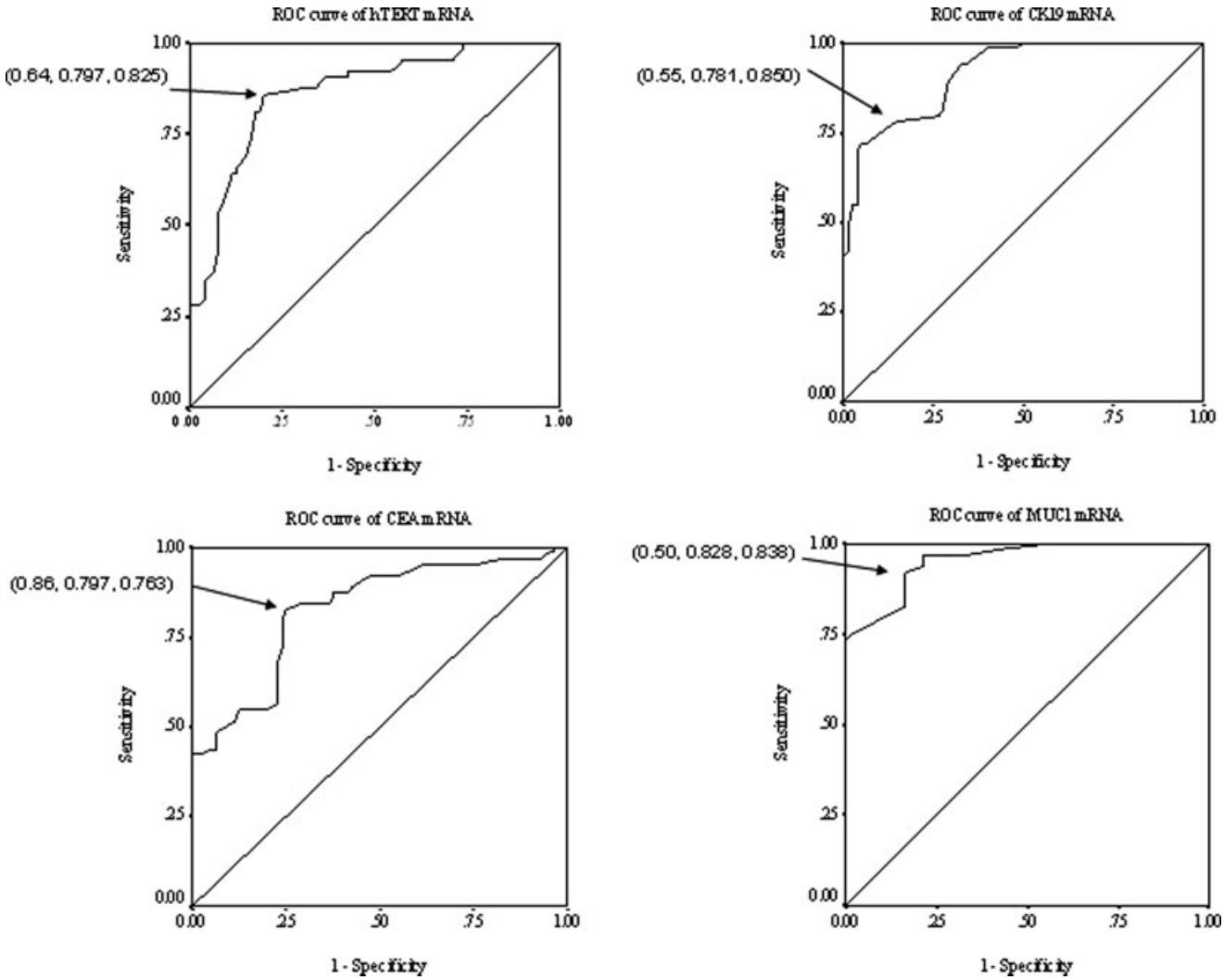

FIGURE 3 – Receiver-operating characteristic (ROC) curves for hTERT, CK19, CEA and MUC1 mRNA from the analysis on membrane-array data of 144 subjects. The sensitivity on the *y*-axis was plotted against the false-positive fraction (1-specificity) on the *x*-axis for various cutoff values. The plot is highlighted with the figures in parenthesis indicating cutoff value, sensitivity and specificity. Area under the ROC curve for hTERT, CK19, CEA and MUC1 is 0.865 (95% CI, 0.805–0.925), 0.916 (95% CI, 0.874–0.959), 0.828 (95% CI, 0.760–0.896) and 0.955 (95% CI, 0.926–0.984), respectively.

Follow-up

All of the patients were carefully followed up regularly at 3-month intervals. At each visit, physical examination, routine blood work-up, serum CEA measurements and liver function tests were conducted as appropriate. Chest X-ray and abdominal ultrasonography were performed every 6 months, and computed tomography or magnetic resonance imaging was carried out if indicated. The development of new recurrent or metastatic lesions following operation was defined as a postoperative metastasis. The postoperative follow-ups lasted until September 2005.

TABLE III – SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE VALUE, NEGATIVE PREDICTIVE VALUE AND ACCURACY FOR EACH OF mRNA MARKERS AND THEIR COMBINATION IN THE DISCRIMINATION BETWEEN GASTRIC CANCER PATIENTS AND HEALTHY INDIVIDUALS

	Sensitivity (%)	Specificity (%)	Positive predictive value (%) (95% confidence interval)	Negative predictive value (%)	Accuracy (%)
hTERT mRNA	79.7 (66.0–93.4)	82.5 (69.5–95.5)	78.5 (64.4–92.5)	83.5 (70.9–96.2)	81.3
CK-19 mRNA	78.1 (64.0–92.2)	85.0 (72.8–97.2)	80.7 (67.2–94.1)	82.3 (70.1–95.8)	81.9
CEA mRNA	79.7 (66.0–93.4)	76.3 (61.7–90.8)	72.9 (57.7–88.0)	82.4 (69.4–95.4)	77.8
MUC1 mRNA	82.8 (69.9–95.7)	83.8 (71.2–96.3)	80.3 (66.7–93.9)	85.9 (74.0–97.8)	83.3
Any 1 mRNA	98.4 (94.2–102.7)	53.8 (36.7–70.8)	63.0 (46.5–79.5)	97.7 (92.6–102.8)	73.6
Any 2 mRNA	95.3 (88.1–102.5)	62.5 (46.0–79.0)	67.0 (51.0–83.1)	94.3 (86.5–102.2)	77.1
Any 3 mRNA	89.1 (78.4–99.7)	91.3 (81.6–100.9)	89.1 (78.4–99.7)	91.3 (81.6–100.9)	90.3
All 4 mRNA	60.9 (44.3–77.6)	100.0	100.0	76.2 (61.7–90.7)	82.6

TABLE IV – CORRELATIONS BETWEEN CLINICOPATHOLOGICAL FEATURES OF GASTRIC CANCER PATIENTS AND DETECTION OF EACH OF 4 mRNA MARKERS

	hTERT			CK-19			CEA			MUC1		
	+	−	*p*	+	−	*p*	+	−	*p*	+	−	*p*
Number	52	12	50	14		53	11		54	10		
Age (<60/≥60 years)	23/29	4/8	0.491	18/32	9/5	0.058	22/31	5/6	0.809	23/31	4/6	0.879
Sex (Male/Female)	32/20	9/3	0.381	32/18	9/5	0.984	33/20	8/3	0.510	35/19	6/4	0.771
Location (C/M/A)[1]	11/8/33	3/2/7	0.943	13/8/29	1/2/11	0.281	14/8/31	0/2/9	0.153	14/9/31	0/1/9	0.118
Tumor size (<5/≥5 cm)	29/23	8/4	0.491	27/23	10/4	0.243	29/24	8/3	0.271	30/24	7/3	0.396
Macroscopic type (Early/Advanced)	3/49	3/9	0.039	2/48	4/10	0.005	2/51	4/7	0.001	3/51	3/7	0.015
Lauren classification (Intestinal/Nonintestinal type)	20/32	6/6	0.463	20/30	6/8	0.847	20/33	6/5	0.302	20/34	6/4	0.174
Depth of tumor invasion (T1/T2/T3/T4)	4/8/32/8	3/2/6/1	0.356	2/9/33/9	5/4/5/0	0.001	2/7/35/9	5/3/3/0	<0.001	2/8/35/9	5/3/2/0	<0.001
Lymph node metastasis (Absent/Present)	15/37	5/7	0.388	10/40	10/4	<0.001	10/43	10/1	<0.001	14/40	6/4	0.033
TNM stage (I/II/III/IV)	7/12/19/14	5/0/4/3	0.074	5/7/21/17	7/5/2/0	<0.001	4/10/22/17	8/2/1/0	<0.001	7/11/19/17	5/1/4/0	0.020
Vessel invasion (Absent/Present)	20/32	7/5	0.209	15/35	12/2	<0.001	17/36	10/1	<0.001	20/34	9/1	0.002
Distant metastasis (Absent/Present)	38/14	9/3	0.892	34/16	13/1	0.063	36/17	11/0	0.028	37/17	10/0	0.038
Postoperative Recurrence/metastasis	20/22	9/3	0.268	27/23	12/2	0.032	28/25	11/0	0.004	25/29	10/0	0.006

[1]C, cardia; M, body; A, antrum.

TABLE V – CORRELATIONS BETWEEN CLINICOPATHOLOGICAL FEATURES OF GASTRIC CANCER PATIENTS AND DETECTION OF 4 mRNA MARKERS IN COMBINATION

	Any 1 marker			Any 2 markers			Any 3 markers			All 4 markers		
	+	−	*p*	+	−	*p*	+	−	*p*	+	−	*p*
Number	63	1		61	3		54	10		39	25	
Macroscopic type (Early/Advanced)	5/58	1/0	0.002	4/57	2/1	<0.001	2/52	4/3	<0.001	1/38	5/20	0.020
Depth of tumor invasion (T1/T2/T3/T4)	6/10/38/9	1/0/0/0	0.041	5/10/37/9	2/0/1/0	0.017	3/9/36/9	4/1/2/0	<0.001	1/4/26/8	6/6/12/1	0.007
Lymph node metastasis (Absent/Present)	19/44	1/0	0.135	17/44	3/0	0.009	14/43	6/3	0.001	6/33	14/11	0.001
TNM stage (I/II/III/IV)	11/12/23/17	1/0/0/0	0.221	10/11/23/17	2/1/0/0	0.103	7/11/22/17	5/1/1/0	0.002	1/8/15/15	11/4/8/2	<0.001
Vessel invasion (Absent/Present)	26/37	1/0	0.238	24/37	3/0	0.038	21/36	6/1	0.013	10/29	17/8	0.001
Distant metastasis (Absent/Present)	46/17	1/0	0.554	44/17	3/0	0.286	40/17	7/0	0.092	24/15	23/2	0.007
Postoperative recurrence/metastasis (Absent/Present)	38/25	1/0	0.420	36/25	3/0	0.155	32/25	7/0	0.025	17/22	22/3	<0.001

With a median follow-up duration of 28 months (range, 20–33 months), the correlations between postoperative metastasis and expression of molecular markers analyzed separately or in combination were explored.

Statistical analysis

All statistical analyses were done by using the Statistical Package for the Social Sciences Version 12.0 software (SPSS, Chicago, IL). ROC curve analyses were performed to analyze membrane array data of hTERT, CK-19, CEA and MUC1 gene expression in the peripheral blood of the subjects. The area under ROC curve (AUC) and the corresponding 95% confidence intervals (CI) were calculated for each marker. The cutoff value at the highest accuracy (minimal false-negative and false-positive results) was determined. On the basis of the calculated cutoff 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. The Chi-square test was used to analyze potential correlation between the expression of markers used separately or in combination and the clinicopathological features of GC patients. To clarify the clinical significance of these mRNA markers combined into a diagnostic panel as the predictor of postoperative metastasis, multivariate adjustment was performed by the logistic regression analysis. The overall survival rates were calculated by the Kaplan–Meier method, and the differences in

TABLE VI – CORRELATIONS BETWEEN DETECTION OF 4 mRNA MARKERS IN COMBINATION AND POSTOPERATIVE RECURRENCE/METASTASIS, USING MULTIVARIATE LOGISTIC REGRESSION ANALYSIS

Variables	β	SE	*p* value	Odds ratio	95% confidence interval
Any 1 marker	0	74.013	1.000	1.000	ND
Any 2 markers	0	52.335	1.000	1.000	ND
Any 3 markers	6.593	30.222	0.827	729.985	ND
All 4 markers	1.867	0.710	0.009	6.471	1.609–26.026

β, coefficient; SE, standard error; ND, not determined.

survival rates were analyzed by log-rank test. A probability of less than 0.05 was considered to be statistically significant.

Results

Figure 1 shows the representative results of membrane array hybridization for a GC patient and a normal person. Significant overexpression of markers was observed in the peripheral blood of GC patient, but not in the control sample. The detection sensitivity of membrane array was estimated to be 25 cancer cells or less per 5 ml of blood (or 5 cancer cells/ml of blood) (Fig. 2). ROC curve analyses on membrane array data of 144 subjects (patients and normal persons) were performed. The ROC curves for each of mRNA markers are shown in Figure 3. According to the ROC curve analyses, the optimal cutoff values and AUC for particular single mRNA markers were as follows: 0.64 and 0.865 (95% CI, 0.805–0.925) for hTERT, 0.55 and 0.916 (95% CI, 0.874–0.959) for CK-19, 0.86 and 0.828 (95% CI, 0.760–0.896) for CEA and 0.50 and 0.955 (95% CI, 0.926–0.984) for MUC1. The examination of single markers with membrane array displayed degrees of sensitivity, specificity and diagnostic accuracy ranging from 78.1% to 82.8%, 76.3% to 85% and 81.3% to 83.3%, respectively (Table III). The combined detection of these 4 markers with membrane array yielded more promising outcomes with sensitivity of 89.1%, specificity of 91.3% and diagnostic accuracy of 90.3% for GC, on the condition that 3 markers of them were overexpressed. Among the 4 markers, CEA and MUC1 mRNA were 2 significant indicators for some clinicopathological characteristics, including macroscopic type of tumor ($p = 0.001$ *vs.* $p = 0.015$), depth of tumor invasion (both $p < 0.001$), lymph node metastasis ($p < 0.001$ *vs.* $p = 0.033$), TNM stage ($p < 0.001$ *vs.* $p = 0.02$), vessel invasion ($p < 0.001$ *vs.* $p = 0.002$), distant metastases ($p = 0.028$ *vs.* $p = 0.038$) and postoperative recurrence/metastases ($p = 0.004$ *vs.* $p = 0.006$) (Table IV). As analyzed in combination, expression of any 3 markers or all the 4 markers in the panel was more significantly correlated with the clinicopathological characteristics than single markers or any 2 markers (Table V). The GC patients with expression of all 4 markers had a relative risk of 6.471 to develop postoperative recurrence/metastasis, compared with those positive for none of the markers (Table VI). Furthermore, statistically significant difference was observed between the GC patients with all 4 markers expression and those without any marker expression, using the log-rank test (Fig. 4*b*; $p = 0.0223$), whereas no significant differences were found between GC patients with any 3 markers expression and those without any 3 marker expression (Fig. 4*a*; $p = 0.3007$). Therefore, a combined panel of these 4 mRNA markers may not only be a promising tool for the detection of CTCs, but also a significant and powerful predictor for the prognosis of GC patients.

Discussion

The discovery of GC biomarkers raises expectations of early, accurate detection as well as more effective monitoring and appropriate treatments for this disease. However, the clinical and prognostic value of circulating molecular markers proposed by far remains a matter of debate. We have demonstrated previously that

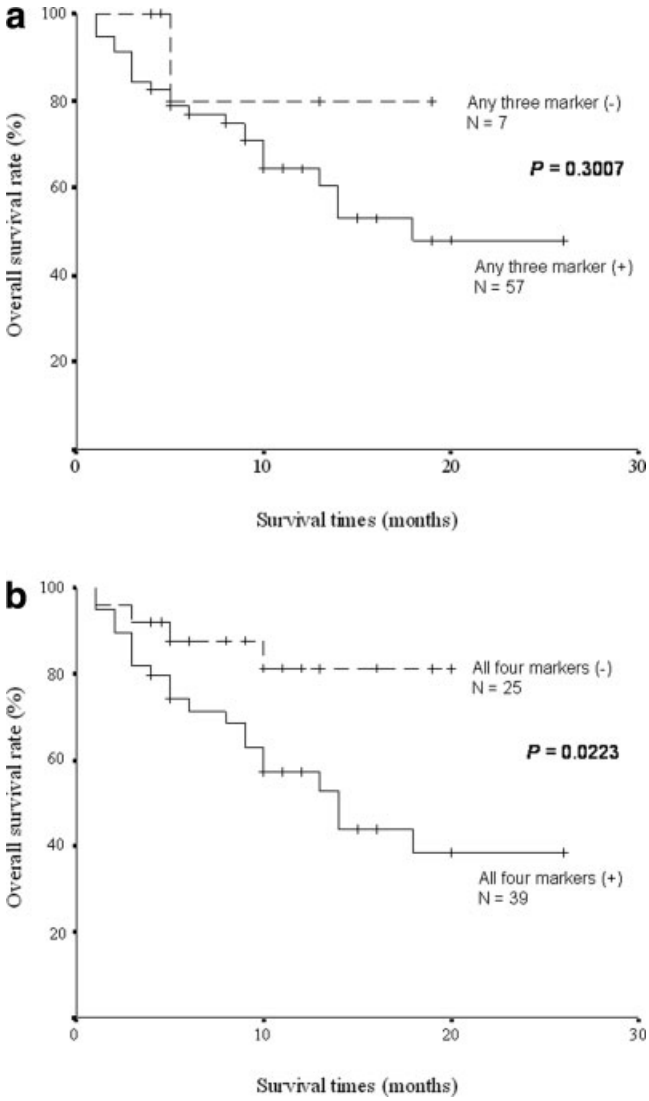

FIGURE 4 – Cumulative overall survival rates of patients with gastric carcinoma by Kaplan–Meier analysis. (*a*) Patients with any 3 mRNA markers expression in the peripheral blood showed no statistical difference in survival when they were compared with patients without any 3 mRNA markers expression, $p = 0.3007$. (*b*) Patients with all 4 mRNA markers expression in the peripheral blood showed significantly poorer survival than those without any 1 mRNA marker expression, $p = 0.0223$.

RT-PCR of CEA mRNA marker was a highly sensitive approach for CTCs detection in GC patients.[25] Additionally, a panel of molecular markers could enhance the sensitivity for CTCs detection, compared with single markers in use.[21] RT-PCR assay is by far regarded widely to be the most sensitive method for detecting tumor-associated molecular markers.[26–32] However, for multiple gene detection, RT-PCR is too time-consuming and laborious to apply in clinical diagnosis. Accordingly, the present study focused on the diagnostic advantage and utility of high-throughput, multimarker membrane array for the detection of CTCs spread from primary GC. For this purpose, the detection rate and diagnostic accuracy of hTERT, CK-19, CEA and MUC1 were assessed separately and in combination for their correlation with clinicopathological features and their potential for predicting postoperative recurrence/metastases.

With membrane array, we observed that the detection rates of using single markers for CTCs in GC patients ranged from 78.1%

to 82.8%, apparently higher than the results with RT-PCR (61.9–78.6%) in our previous observation[25] or those in another investigation (35.5–51.6%).[13] This indicates that this highly sensitive, high-throughput method can serve as an appropriate tool for GC detection. In the literature, the presence of CTCs in the peripheral blood has been reported to be associated with malignant biological properties, more advanced disease and poor prognosis of GC patients.[12–14,30] Similarly, our results showed that the frequency of hTERT, CK-19, CEA and MUC1 mRNA overexpression were significantly higher in advanced GC patients (82.8–87.9%) than in patients with early stage GC (33.3–50%). Moreover, the detection rates for CTCs in GC patients by using single markers CK-19, CEA or MUC1 mRNA were prominently higher in GC patients with stage III and IV GC than in patients with stage I and II GC. These 3 markers were detectable in all patients with stage IV GC. In addition, both CEA and MUC1 mRNA were found in all GC patients with coexisting distant metastasis. Likewise, Mori *et al.* indicated that the positive detection rate for tumor-specific mRNA in peripheral blood samples increased with the advanced stages of gastrointestinal malignancies.[26] After a median follow-up duration of 28 months, 46–47.2% of GC patients positive for CK-19, CEA or MUC1mRNA developed postoperative recurrence/metastases, whereas only 0–14.3% of GC patients expressing none of the 3 markers did. Thus, the detection of CTCs in peripheral blood with membrane arrays can also be used as a powerful prognostic approach for predicting tumor relapse of GC after operation. Among the 4 mRNA markers analyzed, CEA and MUC1 mRNA are suggested to play a more important role in GC staging, monitoring and prognosis than do hTERT and CK-19 mRNA. CEA mRNA has been proposed to be a more reliable marker than transcripts of cytokeratins for the detection of CTCs in peripheral blood of GC patients,[33] which is also in agreement with our findings.

As expected, the detection rate and diagnostic accuracy for CTCs in GC patients were significantly increased, as a panel of multiple markers was employed, compared with those obtained using single markers. According to several recent studies,[34–36] the use of a panel of molecular markers provides a more sensitive method for the detection of several human malignancies including colorectal cancer, lung cancer and breast cancer. In the present study, we demonstrated that with combination of 4 mRNA markers, expression of any 3 mRNA markers could achieve the highest diagnostic accuracy (90.3%) in discrimination between GC patients and normal persons, with sensitivity of 89.1% and specificity of 91.3%, whereas 4 detectable mRNA markers were an independently unfavorable prognostic predictor for GC patients postoperatively. Another evidence demonstrated the prognostic value of these mRNA markers was that GC patients with all 4 mRNA markers expression showed a poorer survival rate than those without any 1 mRNA marker expression. Using the expression of any 3 mRNA markers as positivity criterion, our membrane array-based method with a 4-marker panel displayed a considerable potential for application to GC diagnosis. Concomitant molecular analysis for CTCs with a multimarker panel is justifiable supplementary approaches to current pathological staging system, which may help physicians make accurate judgment on clinical treatments and predicting prognosis for GC patients. The high false positive rate (43.5%) of membrane array in the prediction of postoperative recurrence/metastasis, at least in part, might result from the timing of blood sampling during surgical procedures,[37] which probably enhanced the relief of CTCs. A blood sampling procedure carried out before or after operation can be a resolution to overcome such a problem. However, the optimal sampling time for membrane array remains to be elucidated. In addition, prolonged follow-up period may also decrease its false positive rate in the prediction of postoperative recurrence/metastasis.

In conclusion, we have demonstrated that a multimarker membrane array-based method can serve as a promising tool for both early diagnosis and postoperative surveillance of GC patients with considerably high sensitivity and specificity. Because of the limited follow-up period, nevertheless, the correlation between the identification of CTCs with membrane array and GC patients' survival time remains to be determined before its extensive application.

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