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SHORT COMMUNICATION

The use of multiple molecular markers as predictors of the clinical prognosis of patients with colorectal cancer

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Abstract Serum carcinoembryonic antigen (CEA) is most commonly used as a prognostic biomarker for evaluating curatively resected colorectal cancer (CRC) patients, but it has a low sensitivity and specificity. The aim of this study was to evaluate potential genetic markers in CRC patients using membrane array. Fifty CRC patients were enrolled and mRNA expression in their tissues were analyzed using membrane array analysis. Seven genes were analyzed in this study, including *ATP2A2*, *GLUT1*, *MMP13*, *MAGE-A2*, *MAGE-A7*, *MAGE-A8*, and *MAGE-A12*. Correlations between the results of the membrane array and the clinicopathological features of these CRC patients were then evaluated. The results show that the overexpression of any three or four of these seven genes is correlated with tumor invasion depth, lymphatic invasion, advanced stage, and postoperative recurrence (all $p < 0.005$). Furthermore, the expression of any four genes was more significantly correlated with clinicopathological characteristics than the expression of only two or three genes. The combination of multiple molecular markers and the membrane array method might be useful for predicting postoperative relapse in CRC patients.

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Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world. About 80% of CRC patients receive curative resection, but 40—50% of these patients experience recurrence within 5 years.[1] In fact, 20—25% of patients with stage II CRC develop recurrence.[2] The liver and lungs are the most frequent sites of metastasis in CRC patients.[3] Approximately 80—90% of patients with liver metastasis are diagnosed with unresectable disease.[2] The combination of chemotherapy and targeted therapy elevates the overall survival time to 12—20 months, but the 2-year survival rate is still <40%.[4]

Serum carcinoembryonic antigen (CEA) is most commonly used as a prognostic biomarker in curatively resected CRC patients. However, the weakness of CEA is its low sensitivity and specificity. Because a single biomarker only can provide limited sensitivity and specificity, the combination of multiple biomarkers might be able to improve diagnosis in these patients. Wang et al analyzed the expression levels of four genes in peripheral blood samples from CRC patients, and the sensitivity was 92.4% if any one of the four genes was overexpressed.[5] While all four genes were overexpressed, the specificity was high as 100%. The study by Shariat et al suggested that multiple molecular markers can predict the recurrence of bladder

Table 1 Probe sequences of the mRNA markers used in this study.

Gene	Oligonucleotide sequence (5′ → 3′)
ATP2A2	CATTCAAATACCCCCACAGGACCATTCCACACAATCTGCTTAGCCCGAGT
GLUT1	CAACCCCACTTACTTCTGTCTCACTCCCATCCAAACCTCCTACCCTCAAT
MMP13	TAGAGATCCTCCATTTCATTACTCTAACATTCTTCAATGTGGTTCCAGCC
MAGE-A2	GGATTGTCTCCAAAGAGTGTAGTTGATGGTAGTCGAGAAGCTGGAGGCTC
MAGE-A7	CTGCCCAAAGAAACTGCTCCATCCCATCATACACCATTACACTCAACGCT
MAGE-A8	AGCAGCTTCCTGAGCTTCCAATAGACACTGTGCTCCCTCCCATCATACAG
MAGE-A12	TGATCTTTAGCAAATGGTGCAGGACTTTCACATAGCTGGTTTCAACGAGG
*TB**	GAGTTCACGGATATTGCGTTCGATACTGCTGGCGATGAGTTCGAGGACAT
β-actin	TGCATTGTTACAGGAAGTCCCTTGCCATCCTAAAAGCCACCCCACTTCTCTCTAAGGAGA

*TB**: gene of *Mycobacterium tuberculosis* (negative control).

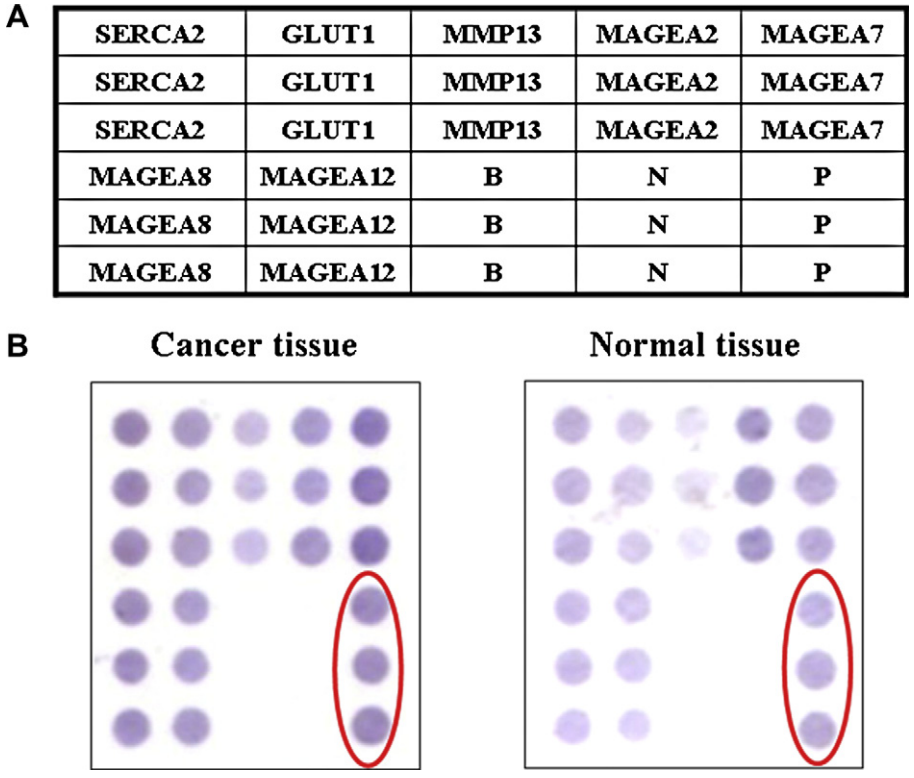

Figure 1 Schematic representation of the membrane array and comparisons of the gene expression patterns between a human colorectal cancer patient and a normal control. (A) Schematic representation of the membrane array with the seven target genes, including *ATP2A2*, *GLUT1*, *MMP13*, *MAGE-A2*, *MAGE-A7*, *MAGE-A8*, and *MAGE-A12*. P: *β-actin* (positive control); N: *Mycobacterium tuberculosis* gene (negative control); B: DMSO (blank control). (B) Gene expression patterns of a colorectal cancer patient (left) and a healthy control (right). The spot within the red circle indicates *β-actin* (positive control).

cancer more significantly than any single molecular marker.[6]

Our recent investigations indicated that the *ATP2A2*, *GLUT1*, *MMP13*, *MAGE-A2*, *MAGE-A7*, *MAGE-A8*, and *MAGE-A12* genes are overexpressed in CRC patients.[7–10] In addition, the use of a multimarker assay based on membrane array analysis, which we had previously developed, demonstrated the advantages of high sensitivity and specificity. Thus, the aim of this study was to analyze a panel of seven molecular markers in CRC patients using the constructed membrane array method and to evaluate their correlation with the clinicopathological features of patients.

Materials and methods

Fifty CRC patients who had undergone surgical resection were recruited from Kaohsiung Medical University Hospital. Total RNA was purified from tumor and normal tissues, and first strand cDNA was synthesized using an Reverse Transcription System (Promega Corporation, Madison, WI, USA). We used VectorNTI software (Life Technologies Corp., Grand Island, NY, USA) to design the sequences of the oligonucleotide probes (Table 1). The newly synthesized oligonucleotide fragments were dissolved in deionized distilled water and then blotted on Nytran SuPerCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate. Dimethyl sulfoxide (DMSO) was also dispensed onto the membrane as a blank control. After rapid drying and cross-linking procedures, the preparation of the gene chip was complete. The procedure of the membrane array was performed based on our previously described protocol.[9] Subsequent quantification analysis of each spot's intensity was carried out using AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA USA). The spots that consistently carried a factor of ≥ 2 were considered to be differentially expressed. For each sample, the membrane array hybridization experiment was performed in triplicate in order to ensure the reproducibility of results. The fold ratio of each gene was calculated as follows: spot intensity ratio = statistical analysis using Statistical Package for the Social Sciences software (version 12.0; SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $p < 0.05$.

Results

Fifty paired tissues (tumor and normal) obtained from the CRC patients were analyzed using the membrane array, the diagram of which is shown in Fig. 1. In the combination

Table 2 Expression levels of multiple genetic markers in tissues obtained from 50 colorectal cancer patients.

	Two genes[#]			Three genes[#]			Four genes[#]		
	P	N	*p*	P	N	*p*	P	N	*p*
Gender									
Female	25	3	0.621	17	11	0.061	12	16	0.166
Male	21	1		19	3		14	8	
Age (y)									
< 60	12	2	0.310		5	0.496		7	1.000
≧60	34	2		27	9		19	17	
Size									
< 5 cm	25	4	0.129	18	11	0.110	12	17	0.093
≧5 cm	21	0		18	3		14	7	
Location									
Colon	34	1	0.075	27	8	0.304	18	17	1.000
Rectum	12	3			6			7	
Depth									
T1 + T2		3	0.004*		4	0.044*		6	0.008*
T3 + T4	43	1		34	10		26	18	
Lymph node metastasis									
Negative	19	3	0.308	11	11	0.004*		17	<0.0001*
Positive	27	1		25	3		21	7	
UICC stage									
I + II	17	3	0.289		11	0.001*		17	<0.0001*
III + IV	29	1		27	3		23	7	
Postoperative metastasis									
Negative	23	2	1.000	14	11	0.025*		16	0.046*
Positive	23	2		22	3		17	8	

* $p < 0.05$.
[#] Indicates any 2, 3, or 4 out of 7 genes showing overexpression.
UICC = International Union Against Cancer.

analysis of the seven genes, the overexpression of any two genes was correlated with tumor invasion depth ($p = 0.004$) and the overexpression of any three or four genes was correlated with tumor invasion depth ($p = 0.044$ and $p < 0.008$, respectively), lymphatic invasion ($p = 0.004$ and $p < 0.0001$), advanced tumor stage ($p = 0.001$ and $p < 0.0001$), and postoperative recurrence ($p = 0.025$ and $p = 0.046$; Table 2).

Discussion

Our developed membrane array demonstrates high sensitivity and specificity,[11] and because of this advantage multiple genetic markers can be analyzed quickly and simultaneously. In this study, we analyzed the expression levels of seven genes in 50 CRC patients. The results indicate that the overexpression of any three of these seven genes is correlated with tumor invasion depth, lymphatic invasion, advanced tumor stage, and postoperative recurrence. Furthermore, the overexpression of any four genes was more significantly correlated with these clinicopathological characteristics than any two or three genes.

The treatment of colorectal cancer mainly consists of surgical resection. However, residual cancer cells left over from surgery may cause micrometastasis, resulting in the patient developing local recurrent or distance metastasis. Therefore, effectively evaluating patient prognosis is very important. The results of this study indicate that the multimarker membrane array we developed demonstrates significant correlation between many clinicopathological characteristics, including tumor invasion depth, lymphatic invasion, and postoperative metastasis.

In recent years, many studies have focused on the correlation between a single mRNA marker and cancer. Although they provided information on the usefulness of many markers, one of the limitations is that that analysis of a single marker can only provide limited sensitivity and specificity.[12–15] Because of the heterogeneous expression of tumor-related genes, it is generally believed that multimarker assays are more reliable, sensitive, and specific than single-marker assay.

In brief, we combined seven genetic markers to construct a new diagnostic platform that uses a membrane array. The expression levels of these genes demonstrate correlations with the clinicopathological characteristics of CRC patients, making these genes potentially useful for determining the prognosis of colorectal cancer patients who have undergone surgical resection. However, additional research on larger patient populations are required in order to evaluate the sensitivity and specificity, and long-term follow-up examinations are needed to confirm the clinical significance of this diagnostic platform.

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