SPANDIDOS PUBLICATIONS

Great potential of a panel of multiple hMTH1, SPD, ITGA11 and COL11A1 markers for diagnosis of patients with non-small cell lung cancer

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Received June 2, 2006; Accepted June 30, 2006

Abstract. Research on molecular mechanisms underlying the carcinogenesis of non-small cell lung cancer (NSCLC) may provide gene targets in critical pathways valuable for improving the efficacy of therapy and survival of patients with NSCLC. However, the molecular markers highly sensitive for the prognosis and treatment evaluation of NSCLC are not yet available. To explore candidates, we conducted an oligonucleotide microarray study with three pairs of NSCLC and normal lung tissue, and determined 8 differentially expressed genes including the Human MutT homologue (hMTH1), Surfactant protein D (SPD), Human hyaluronan binding protein 2 (HABP2), Crystalline-mu (CRYM), Ceruloplasmin (CP), Integrin alpha-11 subunit (ITGA11), Collagen type XI alpha I (COL11A1), and Lung-specific X protein (Lun X). Four lung cancer-related markers MUC-1, hTERT, hnRNP B1, and CK-19 were also incorporated for further analysis. The expression profiles of the twelve genes in seventy pairs of NSCLC tumor and normal lung tissue were then detected quantitatively by using membrane array and quantitative real-time PCR (qRT-PCR). The data of the membrane array and qRT-PCR were compared for consistency and the potential of these mRNA markers in clinical application. The results showed that membrane array and qRT-PCR obtained consistent data for the tested genes in both sensitivity and specificity (correlation coefficient 0.921, $p<0.0001$). For patients' clinicopathological characteristics, the overexpression of hMTH1, SPD, HABP 2, ITGA11, COL11A1, and CK-19 was significantly correlated with the pathological stage ($p<0.05$). In addition, the overexpression of hMTH1, SPD, ITGA11, and COL11A1 was correlated with lymph node metastasis and poor prognosis. This is the first report relating SPD to a prognosis marker for NSCLC. Moreover, the combined detection of these four mRNA markers by membrane array had a sensitivity of 89% and a specificity of 84% for NSCLC, significantly higher than these markers had achieved separately. In conclusion, we identified mRNA markers for NSCLC prognosis and therapy evaluation from differentially expressed genes determined by using micro-array. Further studies are needed to collect the data of the mRNA markers used in clinical practice.

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Key words: non-small cell lung cancer, membrane array, quantitative real-time PCR, mRNA marker, molecular diagnosis

Introduction

Lung cancer is a leading cause of cancer-related death worldwide, and non-small cell lung cancer (NSCLC) accounts for about 80% of newly diagnosed cases of pulmonary malignancy (1). Since the survival rate of NSCLC patients receiving conventional therapies is unsatisfactory, a novel treatment that can effectively improve patients' survival is urgently needed. A number of studies suggest that the choice of therapeutic regimens and prognosis for patients are associated with the expression of certain tumor-specific mRNA markers (2,3). To date, a few mRNA markers have been identified and employed to monitor tumor progression (4). However, the amount of gene transcripts of a particular marker varies greatly among the different NSCLC cells in a tumor as well as among tumors from different individuals. The heterogeneity of marker gene expression in NSCLC cases limits the reliability of a single-marker detection scheme (5). Bret *et al* proposed that a panel of marker genes provides a more reliable and informative approach than a

single-marker assay for the detection of melanoma and breast cancer (6). The application of multiple markers may also serve as a potent mean for NSCLC detection, in turn contributing to better treatment outcomes for NSCLC patients.

The emerging high-throughput technologies, e.g. microarray, do not only offer new insights into the molecular mechanism of tumorigenesis, but also have a great potential to combine with bioinformatics technology, which helps interpret a good deal of gene expression data, into an innovative diagnosis tool (7). However, the data of genomic analysis by microarray in literature is not ready to be used clinically for a number of reasons. (i) The results were based on either relatively small, selected groups of patients or the cases of a big series. It is agreed that the gene expression profiles reported in the relevant studies need to be validated in independent series before being widely used (8,9). (ii) Microarray technology is expensive, thus limiting its availability for many medical settings. (iii) Microarray usually screens a large scale of genes, most of which may not be directly associated with tumor formation from a clinical perspective (10). To provide a solution to this problem we developed a panel of RNA markers comprised of differentially expressed genes derived from NSCLC by a microarray study and four well-established lung cancer-related markers including mucin-1 (MUC-1), telomerase (hTERT), heterogeneous nuclear ribonucleoprotein (hnRNP) B1, and cytokeratin 19 (CK-19). MUC-1, a highly glycosylated glycoprotein, often overexpressed in adenocarcinomas, plays an important role in facilitating invasion and metastasis of malignant cells (11). Telomerase activity is one of the most important prognostic factors for patients with NSCLC, and its potential prognostic implication is independent of tumor stage (12). hnRNP B1, an RNA-binding protein, has been found to be overexpressed in the early stage of lung cancer including bronchial dysplasia, a premalignant lesion of lung squamous cell carcinoma (13). CK-19 is a candidate for a general marker of epithelial cancers (14).

Eight differentially expressed genes were determined by our micrarray analysis. The expression of twelve genes in clinical tissue samples of NSCLC was then detected by membrane array and qRT-PCR, and analyzed for any correlation with patients' clinicopathological characteristics. We identified four mRNA markers as potential indices for NSCLC prognosis and therapeutic evaluation.

Materials and methods

Oligo membrane array preparation. Oligonucleotide microarray [Agilent Human 1A oligo microarray kit (V2), containing probes for 20,227 genes] was performed to analyze the mRNA expression profiles in three tumor/normal tissue pairs of NSCLC patients including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. By using Genespring software 6.1 (Silicon Genetics, Redwood, CA), we obtained 8 genes expressed in the tumors at signal levels 20-fold higher than those in the paired normal tissue. We subsequently conducted oligo membrane array with probes for these 8 genes and additional 4 prognosis-related genes in lung cancer. Visual OMPS (DNA Software, Inc.) was used to design oligonucleotide probe sequences for the target genes and ß-actin which served as an internal control (Table I).

Specimen collection and cDNA preparation. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH). Seventy pairs of tumor and adjacent normal lung tissue were obtained surgically from patients with pathologically proven NSCLC at the Division of Chest Surgery, Department of Surgery, KMUH. One half of the tissue samples was immediately placed in liquid nitrogen for shipment to the laboratory, and then stored at -80˚C until RNA isolation. The other half of the tissue samples was sent for routine histopathological diagnosis. The protocol of RNA extraction and first strand cDNA synthesis was described previously by Wu *et al* (15). RNA concentration was determined spectrophotometrically on the basis of absorbance at 260 nm (Beckman, DU800, USA).

Membrane array analysis.

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 membrane arrays 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-chloro3-indoyl-phosphate (NBT/BCIP) for 15 min. The membrane arrays were then scanned with an Epson Perfection 1670 flat bed scanner (Seiko Epson Co., Nagano-ken, Japan). Subsequent quantification analysis of each spot intensity was carried out by using AlphaEase® FC software (Alpha Innotech Co., San Leandro, CA, USA). For each sample, the experiment of membrane array hybridization was done in triplicate to ensure the reproducibility of the results.

Data analysis. cDNA of 70 lung tissue pairs (NSCLC tissue and adjacent normal lung tissue) were applied to our diagnostic membrane arrays for analysis as described above. The expression (spot intensity) of each gene in the 70 tissue pairs was then normalized to ß-actin and presented as a relative expression ratio. A gene exhibiting an expression ratio two-fold higher in the cancer tissue than that in the paired normal tissue was designated as overexpressed.

Quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was performed in a Rotor-Gene 2070 thermocycler (Corbett Research Inc., Sydney, Australia). Each reaction mixture contained 2 μl of 20 mM dNTP, 2 μl of 30 mM MgCl, 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 (i.e., synthesized dsDNA) were quantified by measuring the fluorescent intensity at the end

rimers for real-time Q-PCR and the oligonucleotides for membrane arrays.

Gene name	Oligonucleotides	Primers (Primers from 5' to 3')
Human MutT homologue (hMTH1)	CAGACAGCAT CCAGGGGACC CCCGTGGAGA GCGACG	f: TAGTCAGCTGTTAGACTCCCTGC r: GTGGAAAGCACACCAACAGG
Surfactant protein D (SPD)	AAAGTTGAGCTCTTCCCAAATGGCCAA AGTGTCGGGGAGAAGATTTTCAA	f: AGAGGAGCCCCAGAAAGCAA r: GCCCAGCTCTTTCCACTGCT
Human hyaluronan binding protein 2 (HABP2)	TCTGGCTGGGGTGTTACAGAAACAGGA AAAGGGTCCCGCCAGCTCCTGGA	f: CATGCGTGCCTTTACTGGAA r: CGTCCGCATCTCGGGTTTCT
Crystalline-mu (CRYM)	TCCCTTCCCACCAGGCTACTGTGCTACT CTTTGAG	f: TCCCTTCCCACCAGGCTACT r: TGGCAGAAACTGCAGCTGTT
Ceruloplasmin (CP)	GAGAATGGATGCTCAGCTGTCAGAATCT AAACCATCTGAAAGCCGGTTTG	f: CCCTGGAGAATGGATGCTCA r: CTAACATGCTTCCCACGGATATT
Integrin alpha-11 subunit (ITGA11)	CAAGAACCATGGTGCATACCTGGGGTA CACAGTCACATCGGTCGTGTCCT	f: GACGGGAGACGTGTACAAGTGTC r: CCGAGGCGCATGTTGTC
Collagen type XI alpha I (COL11A1)	GAACTGTTAACATCGCTGACGGGAAGT GGCATCGGGTAGCAATCAGCGT	f: GACTATCCCCTCTTCAGAACTGTTAAC r: CTTCTATCAAGTGGTTTCGTGGTTT
Lung-specific X protein (Lun X)	CTGCCTGAGTTGGTTCAGGGCAACGTG TGCCCTCTGGTCAATGAGGTTCT	f: CCTCAGTTCTAGGCATGTCCT r: GGGCAGGTTATACTCTCGCT
Heterogeneous nuclear ribonucleoprotein (hnRNP)	AAATTATAACCAGCAACCTTCTAACTACGGT	f: TAACCAGCAACCTTCTAACT r: TATCGGCTCCTCCCACCATAA
Cytokereatin 19 (CK19)	CAACAATTTGTCTGCCTCCAAGGTCCTC TGAGGCAGCAGGCTCTGG	f: ATGAAAGCTGCCTTGGAAGA r: TGATTCTGCCGCTCACTATCAG
Human telomerase reverse transcriptase (hTERT)	AAAGGTGTGCCCTGTACACAGGCGAGG ACCCTGCACCTGGAT	f: AGTGTGTACGTCGTCGAGC r: AGTCCATGTTCACAATCGGC
Mucin-1 (Muc-1)	AATTCCTCTCTGGAAGATCCCAGCACCG ACTACTACCA AGAGCTGCAGAG	f: GTGCCCCCTAGCAGTACCG r: GACGTGCCCCTACAAGTTGG
Bata-actin (ß-actin) (as an internal control)	TCATGAAGTGTGACGTGGACATCCGCA AAGACCTGTACGCCAACACAGTGCTGTC	f: GCATCCACGAAACTACCTTC r: CAGGAGGAGCAATGATCTTG

of each amplification cycle. For each sample, qRT-PCR analysis was repeated in three independent experiments to ensure the reproducibility of the results. Primers used for qRT-PCR are summarized in Table I.

Statistical analysis. The data were analyzed using the Statistical Package for the Social Sciences Version 12.0 (SPSS Inc., Chicago, IL, USA). The two-sided Pearson χ^2 test was used to compare the clinicopathological features and expression patterns of the mRNA markers. The difference between the data obtained by membrane array and qRT-PCR was calculated by using linear regression and the Pearson correlation. ROC curves were constructed by all possible sensitivity/specificity pairs of a particular detection method, determined by continuously varying the cut-off values over the entire range of results obtained (16). The Kaplan-Meier method was used to examine the effect of the overexpression of hMTH1, SPD, ITGA11, and COL11A1 on the overall survival of these 70 patients (17). The log-rank test was used to compare the pairs of Kaplan-Meier curves (18).

Results

Clinicopathological characteristics. Seventy human lung tumor/normal tissue pairs were surgically obtained in this study. Subjects included 42 men and 28 women (average age, 62 years; range, 26-80 years). The pathological types of NSCLC among the patients are: Forty-two (60%) adenocarcinoma, 18 (26%) squamous cell carcinoma, and 10 (14%) large cell carcinoma. The clinicopathological characteristics are summarized in Table II.

Expression of 12 mRNA markers in patients with NSCLC. At first, total RNA was isolated from the paired tissue samples, and then converted to cDNA, sequentially labeled by Dig-dUTP for membrane hybridization and chromogenesis. Fig. 1 shows the results of membrane array hybridization. Fig. 1A charts the corresponding positions of the oligonucleotide probes on membrane array, including the negative controls. Fig. 1B (a) and (b) are representative dot patterns of paired adenocarcinoma and adjacent normal tissue, respectively.

Table II. Clinicopathological characteristics of the NSCLC patients.

	Lung cancer cases N=70
Age, mean (SD)	61.6 (13.3)
Sex (%)	
Male	42 (60)
Female	28 (40)
Cell type (%)	
Adenocarcinoma	42 (60)
Squamous cell carcinoma	18 (25.7)
Large cell carcinoma	10 (14.2)
Stage (%)	
I	26 (37.1)
II	9 (12.9)
III	17 (24.3)
IV	18 (25.7)
Tumor size (%)	
1	17 (24.2)
2	26 (37.1)
3	9 (12.9)
4	18 (25.7)
Metastasis (%)	
0	51 (73)
1	19 (27)
Lymph node (%)	
0	41 (58.5)
1-3	29 (11.5)

Fig. 1B (c) and (d) are representative dot patterns of squamous cell carcinoma and adjacent normal lung tissue, respectively. By comparing the expression patterns, it is evident that all the 12 mRNA markers were overexpressed in adenocarcinoma; and 9 markers in squamous cell carcinoma except for Lun X, hnRNP, and CK-19.

Comparison of membrane array and qRT-PCR. To examine the feasibility of the membrane array in determining the expression level of mRNA markers, 210 paired measurements of HABP2, CRYM, and CK-19 mRNA expression levels using qRT-PCR vs the membrane array in 70 NSCLC patients were analyzed and compared by linear regression and Pearson correlation. It demonstrated a high correlation coefficient of r=0.921 between the two data groups, which was highly significant ($p<0.0001$, Fig. 2). This indicated that there were high degrees of consistency and correlation between the results of real-time Q-PCR analysis and membrane array hybridization.

Differential expression of mRNA markers in paired tissue of NSCLC patients. The mean expression ratios of the 12 mRNA markers in the 70 NSCLC tissue samples were significantly higher than those in the paired adjacent normal lung tissue (2.32 vs 0.59 for hMTH1, 1.57 vs 0.63 for SPD, 2.43 vs 0.64 for HABP2, 2.68 vs 0.52 for CRYM, 1.33 vs 0.43 for CP, 2.79 vs 0.66 for ITGA11, 2.34 vs 0.68 for COL11A1, 2.35 vs 0.55 for Lun X, 1.76 vs 0.49 for hnRNP, 2.79 vs 0.61 for hTERT, 2.11 vs 0.57 for CK-19, and 2.25 vs 0.66 for Muc-1; $p<0.05$) (data not shown). The percentages of overexpressed markers in the 70 NSCLC patients (i.e., a marker whose expression ratio in the cancer tissue was two-fold higher than that in the paired normal tissue were as follows: hMTH1, 74.3% (52/70); SPD, 70% (49/70); HABP2, 77.1% (54/70); CRYM, 81.4% (57/70); CP, 84.3% (59/70); ITGA11, 81.4% (57/70); COL11A1, 86% (60/70); Lun X, 80% (56/70); hnRNP, 70% (59/70); hTERT, 73% (51/70); CK-19, 86% (60/70); and Muc-1, 87% (61/70) (data not shown).

Correlation between expression of mRNA markers and clinicopathological features. No significant correlation was found between the expression levels of the 12 mRNA markers and patients' gender or smoking status (data not shown). The expression levels of hMTH1, SPD, HABP 2, ITGA11, COL11A1, and CK19 (Table III) were significantly correlated with tumor size and stage. In addition, those of hMTH1, SPD, ITGA11, and COL11A1 were correlated with lymph node metastasis ($p\leq0.05$). Therefore, the mRNA markers associated with NSCLC progression were hMTH1, SPD, ITGA11, and COL11A1. ROC curve analysis showed that a particular sample in which ≥2 these four mRNA markers were overexpressed could be determined to be positive. Under such a condition, the sensitivity and specificity of the mRNA marker panel for NSCLC were 89% and 78%, respectively (Fig. 3).

Correlation between expression of mRNA markers and survival of NSCLC patients. Kaplan-Meier analysis revealed that of the 12 mRNA markers, hMTH1, SPD, ITGA11, and COL11A1 could predict poor survival of NSCLC patients (Fig. 4).

Discussion

Our microarray study had identified eight genes expressed in NSCLC tissue at signal levels 20-fold higher than those in the adjacent normal tissue, of which hMTH1, SPD, ITGA11, and COL11A1 could be used as NSCLC prognostic markers. These four markers may even provide a more accurate prediction than does the evaluation of lymph node status. Our data showed that the expression of the four mRNA markers was significantly correlated with lymph node metastasis and poor prognosis. Most of the patients who were positive for these mRNA markers survived <5 years (Table III and Fig. 4). This suggests that the four mRNA markers can potentially serve as indices for NSCLC prognosis and therapeutic outcome in clinical practice. One of the important findings in the present study was the correlation between SPD and lymph node metastasis. To the best of our knowledge, this is the first study relating SPD and NSCLC prognosis. SPD is a collagenous glycoprotein. Each monomer subunit of SPD is composed of an NH_2-terminal region, a long collagenous domain, a neck region, and a highly conserved globular carbohydrate recognition domain that is the functional site for attachment to microorganisms (19). SPD has been described as capable of binding viruses, bacteria, yeast, and mycobacteria as well as altering inflammatory cell responses.

1. hMTH1	2. SPD	3. HABP2	4. CRYM	5. CP	6. ITGA11	7. COL11A1
1. hMTH1	2. SPD	3. HABP2	4. CRYM	5. CP	6. ITGA11	7. COL11A1
1. hMTH1	2. SPD	3. HABP2	4. CRYM	5. CP	6. ITGA11	7. COL11A1
8. LunX	9. hnRNP	10. hTERT	11.CK-19	12.Muc-1	13. control	14.ß-actin
8. LunX	9. hnRNP	10. hTERT	11.CK-19	12.Muc-1	13. control	14.ß-actin
8. LunX	9. hnRNP	10. hTERT	11.CK-19	12.Muc-1	13. control	14.ß-actin

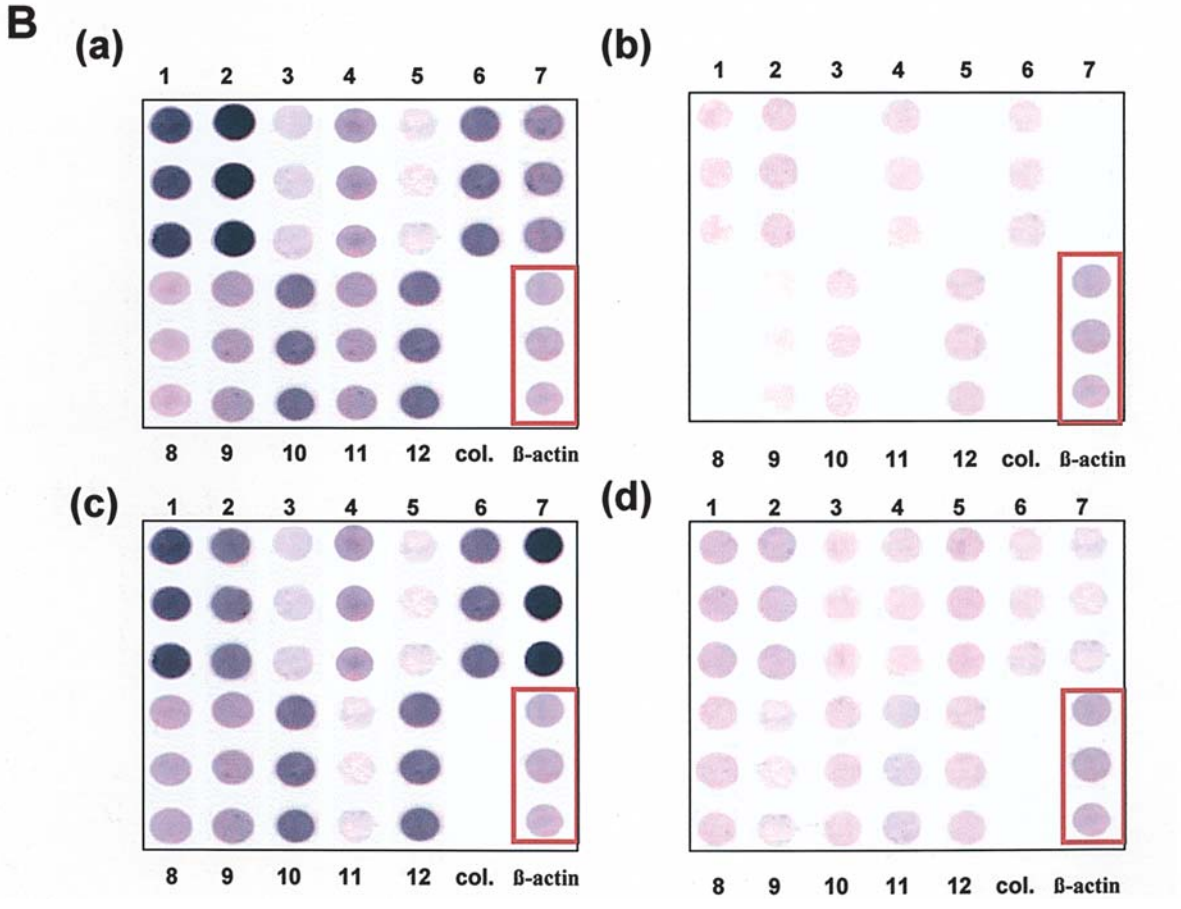

Figure 1. Images of membrane array assay. (A) Positions of oligonucleotide probes on membrane array. hMTH1, SPD, HABP2, CRYM, CP, ITGA11, COL11A1, Lun X, hnRNP, hTERT, CK-19, and Muc-1. (B) Detection of NSCLC and adjacent normal tissue by membrane array. (B) (a) Adenocarcinoma; (b) adjacent normal tissue; (c) squamous cell carcinoma; (d) adjacent normal lung tissue.

Literature review showed that almost all reports on SPD concerned bacterial or viral infection. However, a supervised analysis may be required to identify a subset of the SPD gene, helpful in the differential diagnosis of NSCLC.

As a result, we included hMTH1, SPD, ITGA11, and COL11A1 genes in a panel of gene targets for the evaluation of therapy outcome. ROC curve analysis indicated that a tissue sample could be regarded to be positive as ≥2 of these four mRNA markers were overexpressed. The data of our clinical trial revealed that the combined detection of the four mRNA markers had a sensitivity of 89% and a specificity of 84% for NSCLC, significantly higher than these markers had achieved separately. It is because the biological characteristics of NSCLC are expressed heterogeneously (20). Kennedy *et al* suggested that the hMTH1 gene expression was found only in adenocarcinoma and squamous cell carcinoma (21). Wang *et al* proposed ITGA11 and COL11A1 to be markers for both adenocarcinoma and squamous cell carcinoma. However, they also indicated that different genotypes of ITGA11 and COLLA1 were present in various types of cancer cells, and that the expression levels of COLLA1 were even higher in squamous cell carcinoma than in adenocarcinoma (22).

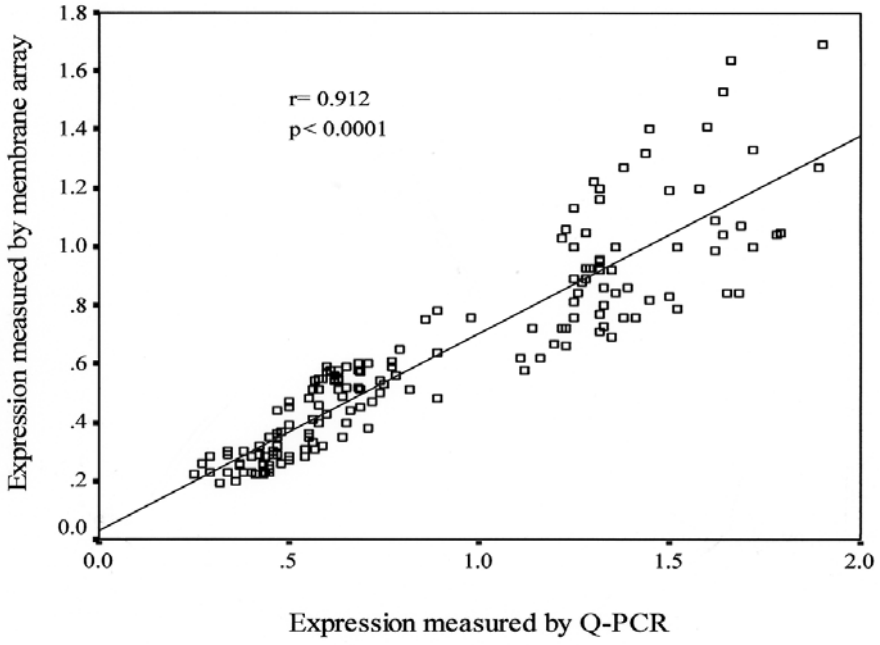

Figure 2. Comparison of membrane array and qRT-PCR. Linear regression and correlation of 210 paired measurements of gene expression for three markers, HABP2, CRYM, and CK-19 in 70 patients with NSCLC by membrane array and qRT-PCR. A high correlation with a coefficient of r=0.912 between membrane array and qRT-PCR was obtained, which was also highly significant (p<0.0001).

Table III. Correlation between clinicopathological features and mRNA marker expression.

	mRNA marker (postive rate %)													
	hMTH1 (%)		SPD (%)		HABP2 (%)		CRYM (%)		ITGA (%)		COL11A1 (%)		CK19 (%)		hTERT (%)	
		P-value		P-value		P-value		P-value		P-value		P-value		P-value		P-value
Cell type		0.213		0.616		0.129		0.092		0.135		0.509		0.060		0.178
AD	29/42 (69)		31/42 (74)		30/42 (71)		33/42 (79)		31/42 (74)		32/42 (76)		30/42 (71)		32/42 (76)	
SQ	16/18 (89)		11/18 (61)		17/18 (94)		18/18 (100)		17/18 (94)		16/18 (89)		17/18 (94)		18/18 (100)	
LA	9/10 (90)		7/10 (70)		8/10 (80)		9/10 (90)		9/10 (90)		9/10 (90)		9/10 (100)		10/10 (100)	
Stage		0.006[a]		0.027[a]		0.016[a]		0.024[a]		0.005[a]		0.036[a]		0.009[a]		0.358
1	16/26 (62)		13/26 (50)		15/26 (58)		22/26 (85)		17/26 (65)		17/26 (65)		16/26 (62)		23/26 (88)	
2	5/9 (56)		6/9 (67)		8/9 (89)		5/9 (56)		6/9 (67)		7/9 (78)		8/9 (89)		7/9 (78)	
3	16/17 (94)		15/17 (88)		15/17 (88)		17/17 (100)		17/17 (100)		16/17 (94)		14/17 (82)		13/17 (76)	
4	17/18 (94)		15/18 (83)		17/18 (94)		16/17 (94)		17/18 (94)		17/18 (94)		18/18(100)		17/18 (94)	
Tumor size		0.002[a]		0.017[a]		0.002[a]		0.136		0.030[a]		0.011[a]		0.004[a]		0.147
1	10/17 (59)		7/17 (41)		10/17 (59)		13/17 (76)		11/17 (65)		10/17 (59)		10/17 (59)		15/17 (88)	
2	17/26 (65)		19/26 (73)		18/26 (69)		21/26 (81)		20/26 (77)		21/26 (81)		19/26 (73)		19/26 (73)	
3	9/9 (100)		7/9 (78)		9/9 (100)		8/9 (89)		8/9 (89)		8/9 (89)		9/9 (100)		9/9 (100)	
4	18/18 (100)		16/18 (89)		18/18 (100)		18/18 (100)		18/18 (100)		18/18(100)		18/18 (100)		17/18 (94)	
Lymph node		0.025[a]		0.005[a]		0.385		0.078		0.031[a]		0.050[a]		0.656		0.178
0	29/41 (71)		22/41 (54)		30/41 (73)		33/41 (81)		29/41 (71)		29/41 (71)		31/41 (76)		35/41 (85)	
1	3/6 (50)		6/6 (100)		4/6 (67)		4/6 (67)		5/6 (83)		6/6 (100)		5/6 (83)		5/6 (83)	
2	9/10 (90)		9/10 (90)		9/10 (90)		10/10 (100)		10/10 (100)		9/10 (90)		8/10 (80)		7/10 (70)	
3	13/13 (100)		12/13 (92)		12/13 (92)		13/13 (100)		13/13 (100)		13/13(100)		12/13 (92)		13/13 (100)	
Metastasis		0.203		0.319		0.053		1.000		0.491		0.097		0.008[a]		0.717
0	37/51 (73)		34/51 (67)		37/51 (73)		44/51 (86)		40/51 (78)		39/51 (76)		37/51 (73)		43/51 (84)	
1	17/19 (89)		15/19 (79)		18/19 (95)		16/19 (84)		17/19 (89)		18/19 (95)		19/19 (100)		17/19 (89)	

[a]p<0.05.

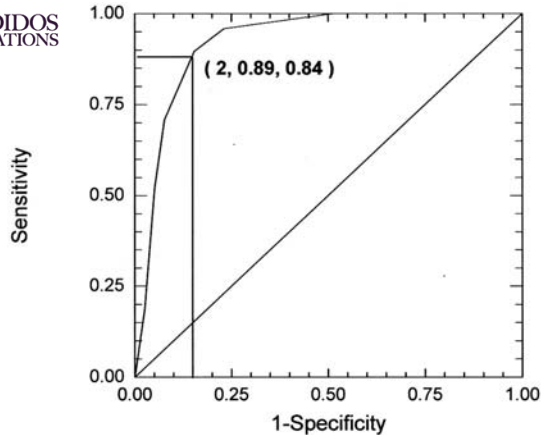

Figure 3. ROC curve. The ROC curve was drawn according to the analysis of our experimental results by using the Statistical Package for Social Sciences Ver. 11.0. An optimal cut-off point was also obtained (0.89, 0.84).

These can serve as the explanations of insufficient sensitivity of a single maker for NSCLC and the variation of single gene expression among the different stages of disease and cell types. A single-marker detection scheme for NSCLC is apt to be greatly affected by the heterogeneity of marker gene expression (5), and thereby its results for therapy evaluation are often controversial and unacceptable.

qRT-PCR has been widely regarded as a sensitive method in specifically detecting the expression of mRNA markers (23). Therefore, we conducted the membrane array and qRT-PCR to evaluate the expression of the 12 genes for comparison. The result of linear regression analysis showed that the data between the membrane array and qRT-PCR were in agreement (r=0.921, p=0.0001). Current gene expression profiling based on bioinformatics tools is highly accurate in the diagnosis of cancer. The gene signature was first described by using microarray technology (24). Although microarray is an excellent tool for initial target discovery, it has been broadly recognized to yield results varying with different users and platforms (25). In the present study, we exploited the membrane array for the merits it provide. Membrane array did not only accurately display the expression level of each single gene, but required only simple devices and materials following easy procedures. The complexity and cost for global gene expression analysis

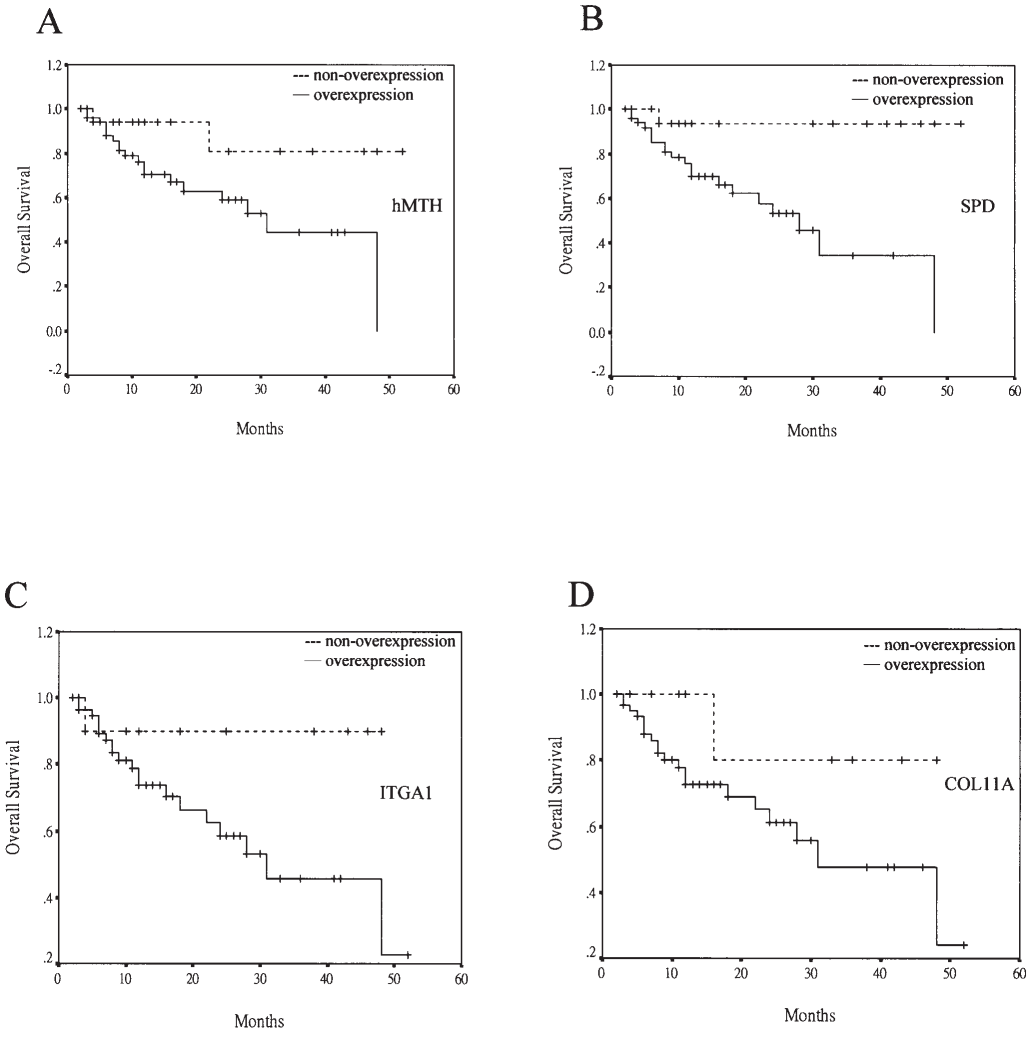

Figure 4. The overall 5-year survival rates for NSCLC patients. (A) NSCLC patients with or without hMTH1overexpression. (B) NSCLC patients with or without SPD overexpression. (C) NSCLC patients with or without ITGA11 overexpression. (D) NSCLC patients with or without COL11A1 overexpression. No significant differences in survival rate existed among the four groups.

could be thus greatly reduced (26,27). For these reasons, the membrane array can be a good choice for detection of gene expression profiles involving limited number of genes (28,29).

In conclusion, we identified mRNA markers for NSCLC prognosis and therapy evaluation from differentially expressed genes determined by using microarray. Further studies are needed to collect the data of the mRNA markers used in clinical practice. The membrane array detection for these markers may serve as a reliable, effective molecular staging method of NSCLC for the use of clinicians.

Acknowledgements

The authors wish to express their thanks to the generous research support provided by Drs T.F. Chen and O.L. Hsu (the founders of Sunrider International), through Give2Asia Foundation, to Kaohsiung Medical University Education and Development Fund. The other is Dr Kuang-His Chang, National Health Research Institutes for his help in performing the statistical analysis.

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