Molecular mechanisms underlying the tumorigenesis of colorectal adenomas: Correlation to activated K-*ras* oncogene

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Abstract. Mutations of K-*ras* gene have been demonstrated in 40-50% of colorectal cancer and large adenoma (>1 cm). This study was intended to clarify the correlation between the existence of K-*ras* oncogene and the pathological features of colorectal adenomas using our recently developed membrane arrays. Moreover, the downstream genes regulated by K-*ras* oncogene were explored to serve as potential biomarkers in the early diagnosis and risk assessment of patients with colorectal adenoma. Specimens were collected from 70 patients with colorectal adenoma. The alterations of K-*ras* oncogene were analyzed by direct sequencing and our constructed membrane arrays, respectively. The results of direct sequencing showed that 21 of 70 samples (30%) had K-*ras* gene mutations. The most frequently mutated sites included codons 12, 13, 15 and 18. Furthermore, activated K-*ras* oncogene was identified in 18 of 70 (25.7%) adenoma by membrane arrays. Statistical analyses showed that the membrane array had the accuracy of 90.0%, sensitivity of 88.9%, and specificity of 90.4%. The frequency of the mutational sites of K-*ras* gene was located as follows: codon 12, 100% (4/4); codon 13, 100% (4/4); codon 15, 75% (6/8); and codon 18, 100% (2/2). The analysis of the correlation between the experimental data and pathological characteristics of adenoma showed that activated K-*ras* oncogenes were significantly associated with the size, number and histology of adenomas (all P<0.001). Finally, we found the downstream genes activated by K-*ras* oncogene, including B-cell CLL/lymphoma 2 (BCL2), *Homo sapiens* H2A histone family, member Z (H2AFZ), *Homo sapiens* RAP1B, member of RAS oncogene family (RAP1B), *Homo sapiens* T-box 19 (TBX19), *Homo sapiens* E2F transcription factor 4, p107/p130-binding (E2F4) and matrix metallopeptidase 1 (MMP1), of which were overexpressed in most of all examined adenomas. These genes were then suggested to have functions involved in cell growth. The preliminary results indicated that the accuracy of membrane arrays was comparable to conventional DNA sequencing in the detection of activated K-*ras* oncogenes. Therefore, we propose that activated K-*ras* oncogene in colorectal adenomas may play an important role in the subsequent colorectal carcinogenesis through a group of K-*ras*-related molecular targets.

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Introduction

Colorectal polyps, especially adenomatous polyps, are widely regarded as pre-malignant lesions. The malignant potential of the colorectal adenomas highly correlated with different grades, depending on their size, shape and histological type (1). Generally, intramucosal carcinoma occurs in 5-10% of colorectal adenomas (2,3). However, those in individuals with family histories of hereditary colorectal adenoma or adenocarcinoma may turn cancerous at a risk of 80% in lifetime (4). In addition, other reports also suggest an increased risk of developing cancer among colorectal adenoma patients, and the possibility of prophylactic effect of polypectomy against subsequent the development of malignancies (5).

K-*ras* is a member of the Ras family, the most common oncogenes activated in a wide range of human cancers. Research on Ras oncogene has established mutations of three members of Ras gene family in various human cancers of tissue and organs, including urinary bladder, mammary gland, rectum, kidney, liver, lung, ovary, pancreas, stomach and the hematopoietic system. The frequency of K-*ras* mutation has been demonstrated to be considerably high in certain types of cancers (6,7), such as in 75% of pancreatic tumors (8,9), 45%

Table I. Nucleotide sequences of primers used for PCR and DNA sequencing.

	Sequences	PCR product (bp)
Exon 1		
Forward	5'-TAATACGACTCACTATAGGGAGATATGTTGAGGGCCCATCTCTC-3'	131
Reverse	5'-TCCTAGGTCAGCGCAACCAAAT-3'	
Exon 2		
Forward	5'-TAATACGACTCACTATAGGGTTCCTACAGGAAGCAAGTAG-3'	148
Reverse	5'-CACAAAGAAAGCCCTCCCCA-3'	
Sequencing primer	5'-CCCTATAGTGAGTCGTATTA-3'	

of colorectal cancers (9), and 48% of lung cancer (10). Some studies have proposed that K-*ras* mutations play a significant role in the onset and progression of colorectal cancer (11). Some others have even pointed out the presence of K-*ras* mutations in ~70% of colorectal cancer and 40% of colonic adenoma >1.0 cm in diameter (12). Martinez *et al* have found that at least one of mutation hotspots such as codons 12 and 13 were mutated in >18% of colonic adenoma >0.5 cm in diameter (13). Factors such as larger size of adenoma, older age, and lower intake of folic acid have been described to be associated with a higher risk of K-*ras* gene mutation (12,14). Individuals with colorectal adenoma >2.0 cm in diameter or bearing K-*ras* mutations were reported to have a higher risk of producing recurrent adenoma during the follow-up period (15). Therefore, the mutation of K-*ras* oncogene is regarded to be a critical step in colorectal tumorigenesis.

According to that described above, K-*ras* gene mutation does not only occur in the early stage of colorectal cancer, but is involved in the transformation of colorectal adenomas. However, studies analyzing the correlation between the molecular mechanisms underlying colorectal adenomas and activated K-*ras* oncogene are not available to date. Therefore, the present study was intended to explore the correlation between the molecular determinants of colorectal adenomas and K-*ras* oncogene. Previously, our laboratory had employed the microarray technique combined with bioinformatics tools to screen all differentially expressed genes after activated K-*ras* oncogene in human adrenocortical cells transfected with a K-*ras* mutant, and determined 22 potential gene targets (16). We also constructed a membrane array specific to these twenty-two genes on nylon membrane, and subsequently carried out a clinical trial in which the membrane array was hybridized with samples from various human cancers. The results indicated that the constructed membrane array had a remarkable potential to detect K-*ras* oncogene in human subjects.

The present study focused on the evaluation of the membrane arrays for determining the presence of activated K-*ras* and serving as a tool to monitor K-*ras* gene mutations in colorectal adenomas, in comparison with DNA sequencing. By analyzing K-*ras* mutations and overexpressed genes activated by K-*ras*, we anticipated to elucidate the roles of the downstream genes regulated by K-*ras* oncogene in the prediction of malignant transformation and clinical significance in colorectal adenomas.

Materials and methods

Specimen collection and DNA extraction. Tissue specimens were obtained from 70 patients with colorectal adenoma at the Kaohsiung Medical University Hospital and Tri-Service General Hospital between March 2004 and February 2005. There were 38 males and 32 females (mean age was 53.1 years, ranging from 40 to 72 years). All tissue samples removed by operation or endoscopic polypectomy, upon acquisition, were frozen instantly in liquid nitrogen, and then stored at -70˚C until analysis. Genomic DNA was isolated using proteinase-K (Stratagene, La Jolla, CA, USA) digestion and phenol/chloroform extraction procedure according to the method by Sambrook *et al* (17).

Total-RNA extraction and first strand cDNA synthesis. The isolation of total-RNAs from tissue specimens was performed with Isogen™ (Nippon Gene Co., Ltd., Toyama, Japan) following the modified acidguanidine thiocyanate and phenol/chloroform extraction method (18). Its concentration was determined spectrophotometrically on the basis of its absorbance at 260 nm. First strand cDNA was synthesized from total-RNA by using a RT-PCR kit. The reverse transcription was carried out in a reaction mixture consisted of 1X transcription optimized 5X buffer, 25 mg/ml oligo(dT)15 primer, 100 mmol/l PCR nucleotide mix, 200 mmol/l M-MLV reverse transcriptase, and 0.5 ml of recombinant RNasin ribonuclease inhibitor (Promega Corp., Madison, WI, USA). 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 4˚C until analysis.

Direct sequencing. To identify the mutations of K-*ras* gene, polymerase chain reaction (PCR) analysis was performed; the oligonucleotide primers for exons 1 and 2 were used, and described in Table I. Briefly, the PCR amplification of DNA samples (20 ng) was carried out in a 50 μl reaction volume with a final concentration of 1X PCR buffer [10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, and 0.01% gelatin], 100 mmol/l each deoxynucleotide triphosphate (Promega), and 5 units (1 unit/μl) of BIOTOOLS DNA polymerase (Biotechnological & Medical Laboratories, S.A., Madrid, Spain) for each reaction. The PCR products were purified by the QIAEX II Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) and then subjected to sequencing using

Table II. Oligonucleotide probe sequences used for membrane arrays.

GenBank identity/Symbol	Sequence of probe
Homo sapiens ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2/ATP2A2	5'-ACCCGGACTTTGAAGGCGTGGATTGTGCAATCTTTGAATCCCCATACCCG-3'
Homo sapiens ATPase, H^{+} transporting, lysosomal 21 kDa, V0 subunit c"/ATP6V0B	5'-CATCGGCCATCGGAACTACCATGCAGGCTACTCCATGTTTGGGGCT-3'
B-cell CLL/lymphoma 2/BCL2	5'-ACAACATCGCCCTGTGGATGACTGAGTACCTGAACCGGCACCTGCACA-3'
Homo sapiens calmodulin 2/CALM2	5'-GAAGCATTCCGTGTGTTTGATAAGGATGGCAATGGCTATATTAGTGCTGCAGAACTTCG-3'
Homo sapiens CCAAT/enhancer binding protein, ß/CEBPB	5'-CCGCCTGCCTTTAAATCCATGGAAGTGGCCAACTTCTACTACGAGGCGGA-3'
Homo sapiens collagen, type IV, α 1/COL4A1	5'-GCAAATGTGACTGCCATGGAGTGAAGGGACAAAAGGGTGAAAGAGGCCTC-3'
Homo sapiens chemokine (C-X-C motif) ligand 11/CXCL11	5'-GTTCAAGGCTTCCCCATGTTCAAAAGAGGACGCTGTCTTTGCATAGGCCC-3'
Homo sapiens chemokine (C-X-C motif) receptor 4/CXCR4	5'-CCCCATCCTCTATGCTTTCCTTGGAGCCAAATTTAAAACCTCTGCCCAGCAC-3'
Homo sapiens cysteine-rich, angiogenic inducer, 61/CYR61	5'-CAGCAGCCTGAAAAAGGGCAAGAAATGCAGCAAGACCAAGAAATCCCCCG-3'
Homo sapiens disheveled, dsh homolog 3 Drosophila)/DVL3	5'-CGTCACCTTGGCGGACTTTAAGGGCGTTTTGCAGCGACCCAGCTATAAGT-3'
Homo sapiens E2F transcription factor 4, p107/p130-binding/E2F4	5'-TGAGATCACAGTGAGTGGCGGCCCTGGGACTGATAGCAAGGACAGT-3'
Homo sapiens v-ets erythro-blastosis virus E26 oncogene homolog 1 (avian)/ETS1	5'-TGGAGCAGCCAGTCATCTTTCAACAGCCTGCAGCGTGTTCCCTCCTATGA-3'
Homo sapiens H2A histone family, member Z/H2AFZ	5'-CGTGGAGATGAAGAATTGGATTCTCTCATCAAGGCTACAATTGCTGGTGGTGGTGTC-3'
Homo sapiens L1 cell adhesion molecule/L1CAM	5'-CCTTCCTGGTGGTGTCCAACACGTCCACCTTCGTGCCCTATGAGATCAAA-3'
Homo sapiens low density lipo-protein-related protein 1/LRP1	5'-ATGCCTGTGAAAACGACCAGTATGGGAAGCCGGGTGGCTGCTCTGACAT-3'
Matrix metallopeptidase 1/MMP1	5'-AAGTGACTGGGAAACCAGATGCTGAAACCCTGAAGGTGATGAAGCAGCCC-3'
Homo sapiens RAP1B, member of RAS oncogene family/RAP1B	5'-GGAAGATGAAAGAGTTGTAGGGAAGGAACAAGGTCAAAATCTAGCAAGACAATGGAA-CAACTGTG-3'
Homo sapiens ribosomal protein L30/RPL30	5'-GCTCCAACTCGTTATGAAAAGTGGGAAGTACGTCCTGGGGTACAAGCAGAC-3'
Homo sapiens solute carrier family 25, member 5/SLC25A5	5'-TCTGATGGGATTAAGGGCCTGTACCAAGGCTTTAACGTGTCTGTGCAGGG-3'
Homo sapiens secreted phospho-protein 1/SPP1	5'-GTGGACAGCCAGGACTCCATTGACTCGAACGACTCTGATGATGTAGATGAC-3'
Homo sapiens TAF12 RNA polymerase II/TAF12	5'-CAGCACCCCTCCACAAGGCTCCATGGCCAATAGTACTGCAGTGGTAAAGA-3'
Homo sapiens T-box 19/TBX19	5'-TCATCTGCTCAATGTGGTGGAGAGTGAGCTTCAGGCAGGGAGGGAAAAAG-3'
ß-actin	5'-TGCATTGTTACAGGAAGTCCCTTGCCATCCTAAAAGCCACCCCACTTCTCTCTAAGGAGA-3'

a double-stranded cycle sequencing system (Gibco-BRL, Gaithersburg, MD, USA). The purified products were then sequenced directly with a T7 promoter/IRD800 (LI-COR, Lincoln, NE, USA), which is a T7 promoter primer (Table I) labeled with a heptamethine cyanine dye, or using DNA polymerase incorporating IRD-labeled dATP for sequencing reaction. Upon completion of the sequencing reaction, 4 μl of formamide loading buffer was added to the reaction mixture,

Table III. Schematic representation of membrane array with 22 genes associated with colorectal adenoma, one housekeeping gene, one tuberculosis gene, and three blank controls.

ATP2A2	ATP6V0B	BCL2	CALM2	CEBPB	MMP1	COL4A1	CXCL11	CXCR4
ATP2A2	ATP6V0B	BCL2	CALM2	CEBPB	MMP1	COL4A1	CXCL11	CXCR4
ATP2A2	ATP6V0B	BCL2	CALM2	CEBPB	MMP1	COL4A1	CXCL11	CXCR4
CYR61	DVL3	E2F4	ETS1	H2AFZ	L1CAM	LRP1	RAP1B	RPL30
CYR61	DVL3	E2F4	ETS1	H2AFZ	L1CAM	LRP1	RAP1B	RPL30
CYR61	DVL3	E2F4	ETS1	H2AFZ	L1CAM	LRP1	RAP1B	RPL30
SLC25A5	SPP1	TAF12	TBX19	TB	Blank	Blank	Blank	ß-actin
SLC25A5	SPP1	TAF12	TBX19	TB	Blank	Blank	Blank	ß-actin
SLC25A5	SPP1	TAF12	TBX19	TB	Blank	Blank	Blank	ß-actin

The symbols represent the genes spotted onto membrane arrays, their GenBank identities are listed in Table II.

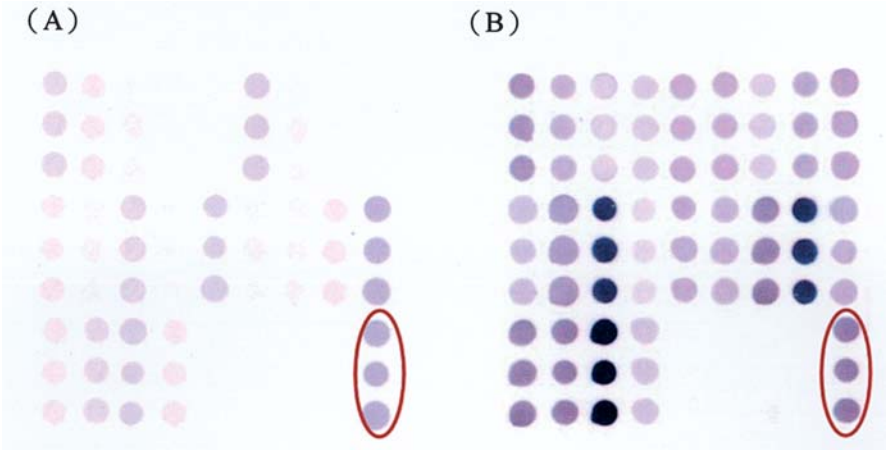

Figure 1. Differential gene expression patterns in the colorectal adenoma patients analyzed by constructed membrane arrays in the detection of activated K-*ras* oncogene. Fourteen of a triplicate set of 22 candidate genes were overexpressed in one colorectal adenoma patient, whereas only one gene was overexpressed in another colorectal adenoma patient. Thus, the results for the former and the latter colorectal adenoma patients were interpreted as positive and negative, respectively. The genes within red circle of each image represent ß-actin (positive control).

the sample heated to 95˚C for 5 min, snap cooled and loaded onto the sequencing gel.

An automated DNA electrophoresis system (Model 4200; LI-COR) with a laser diode emitting at 785 nm and fluorescence detection between 815 and 835 nm was used to detect and analyze the sequencing ladder. Electrophoresis was performed on a 41 cm x 25 cm x 0.2 mm gel consisting of 6% Long Ranger gel matrix (AT Biochem; Malvern, PA, USA) with 7 M urea and a running buffer consisting of 133 mM Tris base, 44 mM boric acid and 2.5 mM EDTA, pH 9.0 at 50˚C. From each sequencing reaction, 1.5 μl of the sample was loaded on each lane. Following the loading of samples, electrophoresis was carried out at a constant voltage of 2000 V with the gel heated to 50˚C. Data collection and image analysis was performed by an IBM486 (Model 90) using the Base Image IR software supplied with the model 4200 DNA sequencer.

Oligonucleotide membrane array preparation. The procedure for the design and preparation of membrane arrays was according to our previously described method (16,19). Using a version of OMP (Oligonucleotide Modeling Platform, DNA Software, Ann Arbor, MI, USA), DNA software tailored to probe sequences for each target are designed and a single best candidate is selected, with ß-actin served as an internal control (Table II). The newly synthesized oligonucleotide fragments were dissolved in DI-water to a concentration of 20 mM and then applied to a BioJet Plus 3000 nanoliter dispense system (BioDot Inc., Irvine, CA, USA), which blotted sequentially the 51 target DNAs (Table I), 1 housekeeping gene (ß-actin), and 1 tuberculosis gene (TB; 50 nl per spot and 1.5 mm between spots) on Nytran® SuperCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) and then cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene) in triplicate. Each spot consists of 20 ng of PCR-amplified DNA derived from sequence-verified cDNA clones. DMSO was also dispensed onto the membrane as a blank control (Table III, Fig. 1).

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

Table IV. The correlation between the membrane arrays and DNA sequencing for the detection of activated K-*ras* oncogene in colorectal adenoma patients.

	Membrane-array		
	Positive	Negative	P-value
Direct sequencing			
Positive	16	2	<0.001
Negative	5	47	

Sensitivity: 88.9% (95% CI, 78.2-99.6%); specificity: 90.4% (95% CI, 80.3-100.4%); positive predictive value: 76.2% (95% CI, 61.7-90.7%); negative predictive value: 97.9% (95% CI, 89.2-102.7%); and accuracy: 90.0%.

Table V. The distribution and detection frequency of K-*ras* mutational site identified by DNA sequencing in 18 colorectal adenoma tissues further analyzed by membrane arrays.

Mutational sites	Number detected by DNA sequencing (%)	Simultaneously positive in membrane arrays (%)
Codon 12	4 (22.2)	4 (100)
Codon 13	4 (22.2)	4 (100)
Codon 15	8 (44.4)	6 (75)
Codon 18	2 (11.2)	2 (100)

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 ExpressHyb Hybridization Solution (BD Biosciences, Palo Alto, CA, USA) containing DIG-11-UTP-labeled cDNA probes, and then incubated with an 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 then exposed to light that excites the light. For signal detection, the membrane arrays were incubated for 15 min in a chromogen solution containing nitroblue-tetrazolium and 5-bromo-4-chloro3-indolyl-phosphate (NBT/BCIP). The hybridized 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 differing by a factor of two or more were considered as differentially expressed.

Statistical analysis. The data were analyzed using the Statistical Package for the Social Sciences Ver. 11.5 software (SPSS Inc., Chicago, IL, USA). The χ^2 test with Yates' correction and the Fisher exact test were used to compare pathological parameters between patients with K-*ras* mutants and with wild-type. A P-value <0.05 was considered to be statistically significant.

Results

The results of direct sequencing showed that of 70 colorectal adenomas, 18 (25.7%) had K-*ras* gene mutations. These mutations were most frequently localized at codons 12, 13, 15 and 18. The criterion for a positive result of the membrane array described in the previous study was determined by using the ROC (receiver-operating characteristic) curve analysis (16): for all 22 target genes, a cut-off value for the number of 11 positive genes was used, where the sensitivity was 88.9% (95% CI, 78.2-99.6%), and the specificity 90.4% (95% CI, 80.3-100.4%) for membrane arrays in the detection of activated K-*ras* oncogene (Table IV). Of 70 colorectal adenomas, 21 (30%) were positive for the membrane array analysis, while 49 (70%) were negative. The results showed that 16 of 21 membrane array-positive specimens was subsequently considered as K-*ras* mutations by direct sequencing, while 47 of 49 membrane array-negative samples was regarded as no K-*ras* gene mutations by direct sequencing. In addition, there were 5 false positives and 2 false negatives, and a remarkable accuracy of 90% in this analysis.

Eighteen specimens with K-*ras* gene mutations by direct sequencing were further analyzed for mutational sites. Four mutation hotspots were thus found, including codons 12, 13, 15 and 18, with the corresponding mutation frequencies of 22.2, 22.2, 44.5 and 11.1%, respectively (Table V). In addition, most adenomas with K-*ras* mutations at codon 12, 13 or 15 had tubulovillous or villous architecture in morphology, multiple in number and a larger tumor size (>1.5 cm), while those with K-*ras* codon 18 mutation were mostly tubular, single and smaller (<1.5 cm) (data not shown). The correlation between mutational sites and results of membrane array was explored. It was revealed that 100% (4/4) of samples with K-*ras* codon 12 mutation, (4/4) of samples with K-*ras* codon 13 mutation, 75% (6/8) of samples with K-*ras* codon 15 mutation, and 100% (2/2) of sample with K-*ras* codon 18 mutation were interpreted as positive using the membrane array analysis.

Pathological characteristics such as size, number, and histology of adenomas were then analyzed for their correlation with presence of activated K-*ras* oncogene by membrane arrays (Table VI). It was revealed that the positive rates of activated K-*ras* oncogene in patients with adenomas >1.5 cm in diameter or multiple adenomas was significantly higher than those in patients with adenomas <1.5 cm in diameter or single adenoma (both P<0.001). In histological analysis, the activated K-*ras* oncogene was found more often in tubulovillous and villous adenomas, whereas wild-type K-*ras* was observed more frequently in tubular adenomas (P<0.001).

When analyzing the most important up-regulated genes activated by K-*ras* in colorectal adenomas, we found that six genes including B-cell CLL/lymphoma 2 (BCL2), *Homo sapiens* H2A histone family, member Z (H2AFZ), *Homo sapiens* RAP1B, member of RAS oncogene family (RAP1B), *Homo sapiens* T-box 19 (TBX19), *Homo sapiens* E2F transcription factor 4, p107/p130-binding (E2F4) and matrix metallopeptidase 1 (MMP1), were prominently over-expressed. The analysis of pathological features showed that

Table VI. The correlation between the membrane arrays for the detection of activated K-*ras* oncogene and pathological features in colorectal adenoma patients.

Variables	Membrane arrays Positive (%) (N=21)	Negative (%) (N=49)	P-value
Maximum size			
<1.5 cm	4 (19)	38 (77.6)	<0.001
≥1.5 cm	17 (81)	11 (22.4)	
Number			
Single	2 (9.5)	37 (75.5)	<0.001
Multiple	19 (90.5)	12 (24.5)	
Histology			
Tubular	3 (14.3)	35 (71.4)	<0.001
Tubulovillous	10 (47.6)	12 (24.5)	
Villous	8 (38.1)	2 (4.1)	

the overexpression of these 6 genes were more frequently encountered in larger adenoma (>1.5 cm), multiple adenomas, and tubulovillous or villous types (Table VII).

Discussion

In the present study, we used membrane arrays detecting downstream genes activated by K-*ras* oncogene to indicate the activation of K-*ras* oncogene. A positive result in the membrane array analysis proposed the existence of activated K-*ras* oncogene. The comparison between data of membrane arrays and direct sequencing showed the membrane array method had higher specificity, sensitivity and accuracy for the detection of K-*ras* activation. The statistical analysis demonstrated the consistence of both methods. This finding also served as evidence that the membrane array was feasible to detect activated K-*ras* oncogene in human tissue, in addition to blood samples (16). The conventional mean for analyzing the oncogenicity of K-*ras* requires DNA sequencing (20). DNA sequencing provides information on gene mutation sites, but it is relatively difficult to determinate in a series of complicated protein activity assays to determine whether or not K-*ras* is activated (21). Using the membrane array analysis, we could effectively carry out the detection of K-*ras* oncogene activation with the advantage of both time-saving and cost-effectiveness, thus suggesting that this convenient method has potential for clinical practice in the future.

Moreover, direct sequencing revealed that the mutation hotspots of K-*ras* gene in colorectal adenomas were codons 12, 13, 15 and 18 (22-24). This finding was consistent with the K-*ras* mutation hotspots in Taiwanese colorectal cancer patients reported previously (24). Our observations suggest that K-*ras* gene in both colorectal cancer and colon adenoma in Taiwanese patients are frequently mutated at codon 15 and to a lesser degree at codons 12, 13 and 61 as documented in the literature (25). The possible explanation might include the exposure to certain carcinogenic agents in different environments favorable for mutations at specific mutation sites (26).

Furthermore, we have demonstrated that the incidence of activated K-*ras* oncogene is significantly associated with the size, number and pathological types of colorectal adenomas. Recent studies have also found that K-*ras* gene mutations are apt to take place in more advanced histology and progression of adenomas (27,28). Cells with activated K-*ras* oncogene are characterized by traits related to cancer, such as lack of contact inhibition, and resistance to apoptosis (29-31). It is reasonable to conjecture that larger or multiple or those of pathologically more advanced adenomas are at relatively high risks of activated K-*ras* mutation, and may cause subsequent transformation. Consequently, it is clear that K-*ras* oncogene plays an important role in deciding the fate of colorectal adenomas. The literature has also showed some association between mutation sites of K-*ras* gene and the

Table VII. The distribution between the six most frequently overexpressed genes in membrane arrays and pathological features in 18 colorectal adenoma patients with activated K-*ras* oncogene.

	BCL2		H2AFZ		RAP1B		TBX19		E2F4		MMP1	
	+	-	+	-	+	-	+	-	+	-	+	-
Maximum size												
<1.5 cm	3	0	2	1	3	0	2	1	2	1	2	1
≥1.5 cm	15	0	15	0	15	0	15	0	14	1	14	1
Number												
Single	2	0	1	1	2	0	1	1	1	1	1	1
Multiple	16	0	16	0	16	0	16	0	15	1	15	1
Histology												
Tubular	3	0	2	1	3	0	3	0	3	1	2	1
Tubulovillous	9	0	9	0	9	0	9	0	9	0	8	1
Villous	6	0	6	0	6	0	6	0	4	1	5	0

degree of cell transformation (32-34). For instance, cells with K-*ras* codon 12 mutation have an increased transforming capacity (35) and reduced apoptosis (36,37), compared to cells with K-*ras* codon 13 mutation; codon 12 mutation is frequently present in metastatic colorectal cancer (32,38), while codon 13 mutation is found in most non-invasive cancers. The results indicate that K-*ras* codon 12 mutation may make significant contribution to cell transformation and malignancy. Consistent with previous investigations, we have also provided evidence that K-*ras* codon 12 mutation were detectable in larger or multiple adenomas or those of pathologically advanced types. However, the consequences of K-*ras* gene mutations at codon 15 and 18, however, remain to be clarified.

The gene expression analysis showed that K-*ras* oncogene regulated downstream genes including BCL2, H2AFZ, RAP1B, TBX19, E2F4 and MMP1 were highly expressed in most colorectal adenoma samples. These genes were known to prominently participate in cell growth and differentiation (39-45). Therefore, we propose that these six genes may significantly contribute to the mechanism underlying tumorigenesis of colorectal adenomas, and thereby can serve as potential markers indicative of the degree of advanced histology. A careful follow-up is advisable for colorectal adenoma patients with these overexpressed genes.

In summary, our constructed membrane array specialized for the detection of activated K-*ras* oncogene seems to be useful, reliable, and convenient; moreover, it affords an additional route to elucidate the molecular pathway of K-*ras* gene activation in colorectal tumorigenesis. Further prospective studies will be required to determine whether the presence of activated K-*ras* oncogene is associated with clinical progression, and recurrence of colorectal adenomas will need the enrollment of a larger patient population and serial follow-up.

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