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

JAW-YUAN WANG1, YUNG-HSIN WANG2, SHU-WEN JAO3, CHIEN-YU LU4, CHAO-HUNG KUO4,5, HUANG-MING HU4,5, JAN-SING HSIEH1, INN-WEN CHONG4,5, TIAN-LU CHENG1 and SHIU-RU LIN1,6,7

1Department of Surgery, Faculty of Medicine, College of Medicine, and Kaohsiung Medical University Hospital, Kaohsiung Medical University; 2Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung; 3Colon and Rectal Surgery Division, Tri-Service General Hospital, Taipei; 4Department of Internal Medicine, Faculty of Medicine, College of Medicine, and Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung; 5Kaohsiung Municipal Hsiao-Kang Hospital; 6Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan

Key words: tumorigenesis, adenoma, K-ras

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 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% 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.

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%
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 MgCl2, 50 mmol/l KCl, and 0.01% gelatin], 100 mmol/l each deoxyribonucleotide 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 I. Nucleotide sequences of primers used for PCR and DNA sequencing.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequences</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>Forward 5'-TAATACGACTCATATAGGGAGATATGGTGAGGCCCATCTC-3'</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TCTTAGGTAGGGCAACAAAT-3'</td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td>Forward 5'-TAATACGACTCATATAGGGTTTCTACAGGAAGCAAGTAG-3'</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CACAAAGAAAGCCCTCCCCA-3'</td>
<td></td>
</tr>
<tr>
<td>Sequencing primer</td>
<td>5'-CCCTATAGTGATCGTATTA-3'</td>
<td></td>
</tr>
</tbody>
</table>
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.
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>
<thead>
<tr>
<th>ATP2A2</th>
<th>ATP6V0B</th>
<th>BCL2</th>
<th>CALM2</th>
<th>CEBPB</th>
<th>MMP1</th>
<th>COL4A1</th>
<th>CXCL11</th>
<th>CXCR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP2A2</td>
<td>ATP6V0B</td>
<td>BCL2</td>
<td>CALM2</td>
<td>CEBPB</td>
<td>MMP1</td>
<td>COL4A1</td>
<td>CXCL11</td>
<td>CXCR4</td>
</tr>
<tr>
<td>ATP2A2</td>
<td>ATP6V0B</td>
<td>BCL2</td>
<td>CALM2</td>
<td>CEBPB</td>
<td>MMP1</td>
<td>COL4A1</td>
<td>CXCL11</td>
<td>CXCR4</td>
</tr>
<tr>
<td>CYR61</td>
<td>DVL3</td>
<td>E2F4</td>
<td>ETS1</td>
<td>H2AFZ</td>
<td>L1CAM</td>
<td>LRP1</td>
<td>RAP1B</td>
<td>RPL30</td>
</tr>
<tr>
<td>CYR61</td>
<td>DVL3</td>
<td>E2F4</td>
<td>ETS1</td>
<td>H2AFZ</td>
<td>L1CAM</td>
<td>LRP1</td>
<td>RAP1B</td>
<td>RPL30</td>
</tr>
<tr>
<td>SLC25A5</td>
<td>SPP1</td>
<td>TAF12</td>
<td>TBX19</td>
<td>TB</td>
<td>Blank</td>
<td>Blank</td>
<td>Blank</td>
<td>ß-actin</td>
</tr>
<tr>
<td>SLC25A5</td>
<td>SPP1</td>
<td>TAF12</td>
<td>TBX19</td>
<td>TB</td>
<td>Blank</td>
<td>Blank</td>
<td>Blank</td>
<td>ß-actin</td>
</tr>
<tr>
<td>SLC25A5</td>
<td>SPP1</td>
<td>TAF12</td>
<td>TBX19</td>
<td>TB</td>
<td>Blank</td>
<td>Blank</td>
<td>Blank</td>
<td>ß-actin</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Mutational sites</th>
<th>DNA sequencing (%)</th>
<th>Simultaneously positive in membrane arrays (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codon 12</td>
<td>4 (22.2)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Codon 13</td>
<td>4 (22.2)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Codon 15</td>
<td>8 (44.4)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>Codon 18</td>
<td>2 (11.2)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

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%.

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 chromogenic 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 $\chi^2$ 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, 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 were 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...
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. The conventional method for analyzing the oncogenicity of K-ras requires DNA sequencing. DNA sequencing provides information on gene mutation sites, but it is relatively difficult to determine in a series of complicated protein activity assays to determine whether or not K-ras is activated. 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. This finding was consistent with the K-ras mutation hotspots in Taiwanese colorectal cancer patients reported previously. 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. The possible explanation might include the exposure to certain carcinogenic agents in different environments favorable for mutations at specific mutation sites.

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. Cells with activated K-ras oncogene are characterized by traits related to cancer, such as lack of contact inhibition, and resistance to apoptosis. 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...
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, H2AZF, 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.

Acknowledgements

The authors are grateful for the generous research support provided by Dr T.F. Chen and O.L. Hsu (the founders of Sunrider International), through Give2Asia Foundation, to Kaohsiung Medical University Education and Development Fund.

References