Potential prognostic and predictive value of UBE2N, IMPDH1, DYNC1LI1 and HRASLS2 in colorectal cancer stool specimens

YU-NUNG CHEN[1*], CHENG-YEN SHIH[2*], SHU-LIN GUO[3-5], CHIH-YI LIU[3,6], MING-HUNG SHEN[3,7,8], SHIH-CHANG CHANG[1], WEI-CHI KU[3], CHI-CHENG HUANG[9,10*] and CHI-JUNG HUANG[11,12*]

[1]Division of Colorectal Surgery, Department of Surgery, Cathay General Hospital, Taipei 10630; [2]Division of Gastroenterology, Department of Internal Medicine, Sijhih Cathay General Hospital, New Taipei 22174; [3]School of Medicine, College of Medicine, Fu Jen Catholic University, New Taipei 24205; [4]Department of Anesthesiology, Cathay General Hospital, Taipei 10630; [5]Department of Anesthesiology, Tri-Service General Hospital and National Defense Medical Center, Taipei 11490; [6]Division of Pathology, Sijhih Cathay General Hospital, New Taipei City 22174; [7]Department of Surgery, Fu Jen Catholic University Hospital, New Taipei 24352; [8]PhD Program in Nutrition and Food Science, College of Medicine, Fu Jen Catholic University, New Taipei 24205; [9]Comprehensive Breast Health Center, Department of Surgery, Taipei Veterans General Hospital, Taipei 11217; [10]Institute of Epidemiology and Preventive Medicine, College of Public Health, National Taiwan University, Taipei 10090; [11]Department of Medical Research, Cathay General Hospital, Taipei 10630; [12]Department of Biochemistry, National Defense Medical Center, Taipei 11490, Taiwan, R.O.C.

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Abstract. Colorectal cancer (CRC) is the most common gastrointestinal malignancy worldwide. The poor specificity and sensitivity of the fecal occult blood test has prompted the development of CRC-related genetic markers for CRC screening and treatment. Gene expression profiles in stool specimens are effective, sensitive and clinically applicable. Herein, a novel advantage of using cells shed from the colon is presented for cost-effective CRC screening. Molecular panels were generated through a series of leave-one-out cross-validation and discriminant analyses. A logistic regression model following reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry was used to validate a specific panel for CRC prediction. The panel, consisting of ubiquitin-conjugating enzyme E2 N (UBE2N), inosine monophosphate dehydrogenase 1 (IMPDH1), dynein cytoplasmic 1 light intermediate chain 1 (DYNC1LI1) and phospholipase A and acyltransferase 2 (HRASLS2), accurately recognized patients with CRC and could thus be further investigated as a potential prognostic and predictive biomarker for CRC. UBE2N, IMPDH1 and DYNC1LI1 expression levels were upregulated and HRASLS2 expression was downregulated in CRC tissues. The predictive power of the panel was 96.6% [95% confidence interval (CI), 88.1-99.6%] sensitivity and 89.7% (95% CI, 72.6-97.8%) specificity at a predicted cut-off value at 0.540, suggesting that this four-gene panel testing of stool specimens can faithfully mirror the state of the colon. On the whole, the present study demonstrates that screening for CRC or cancer detection in stool specimens collected non-invasively does not require the inclusion of an excessive number of genes, and colonic defects can be identified via the detection of an aberrant protein in the mucosa or submucosa.

Correspondence to: Dr Chi-Cheng Huang, Comprehensive Breast Health Center, Department of Surgery, Taipei Veterans General Hospital, No. 201, Section 2, Shipai Road, Taipei 11217, Taiwan, R.O.C.
E-mail: cchuang29@vghtpe.gov.tw

Dr Chi-Jung Huang, Department of Medical Research, Cathay General Hospital, No. 280, Section 4, Renai Road, Taipei 10630, Taiwan, R.O.C.
E-mail: aaronhuang@cgh.org.tw

*Contributed equally

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Introduction

Gastrointestinal cancers encompass a variety of malignant diseases, with only colorectal cancer (CRC) ranked among the most common tumors (1). The integrated conventional staging system (T, tumor size; N, lymph node status; M, distant metastasis) and molecular classifications of CRC may aid in reliable personalized treatments and may contribute to the prediction of the prognosis of cancer (2-4) Moreover, the success of CRC screening and detection must depend significantly on molecular parameters and not exclusively on clinical stage (5,6).

Based on findings concerning peripheral blood possibly reflecting changes that occur in tissues (7,8), numerous molecular parameters from blood specimens have been reported for

CRC detection (9-11). As this type of test does not directly include samples of colonic cell origin, the molecular parameters of numerous genes should be composed to achieve adequate sensitivity and specificity (9,11). Compared with the results of other groups that have used blood specimens for screening, a previous study by the authors involving the extraction of expression profiles from stool specimens revealed a good association of these profiles with CRC progression and recurrence (12-14). The direct detection of changes in gene expression in colonic tissues may contribute to a further understanding of CRC progression and may allow the development of biomarkers and drug targets for this malignant disease (15). Human stool has been studied for CRC screening for several years (16-18), and several lines of evidence have indicated that cells shed from the colonic tract may reflect localized diseases (19-21). Thus, either DNA or RNA extracted from stool specimens can be used to detect colorectal neoplasia accurately (22-24). Genes that are actively expressed in human stool specimens have emerged as specific molecular signatures of CRC (25,26). These genetic molecules may aid in the understanding of the process of the development of CRC (27,28). Moreover, the signatures concurrently reflect CRC biology, and inform prognoses and treatment responses in a non-invasive manner (29). The gene expression status of colonic cells that pass into stool specimens has been considered to faithfully represent CRC manifestations (30-33).

Herein, the novel, to the best of our knowledge, advantage of using cells shed from the colonic tract for cost-effective CRC screening is presented. Previously, genes expressed in human stool specimens that were used to identify patients with differentially staged CRC distinguishing them from healthy donor control samples were acquired using whole-genome microarrays. Expressed genes were further filtered via custom-made microarrays. Specific gene sets were tested with a small number of testing samples using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses, trained with other samples set by a series of leave-one-out cross-validation (LOOCV) and discriminant analyses (34,35), and then confirmed with the third sample set for the probabilities of group membership (healthy donor or CRC). The corresponding proteins of target genes were assessed based on CRC tissue arrays. Furthermore, a logistic regression model was used to predict diseases for a specific molecular panel (36,37).

Materials and methods

Study participants and ethical approval. Human stool specimens were collected from 29 healthy donor controls (age range, 23-78 years; 8 males and 21 females) and 58 patients with well-diagnosed CRC (age range, 29-87 years; 33 males and 25 females) at the Sijhih Cathay General Hospital for predictive model training. All the participating subjects provided their written informed consent. The research was conducted with the obtained approval (approval no. CGH-P101014) of the Institutional Review Board of the Cathay General Hospital and according to the Principles of the Declaration of Helsinki.

All 87 cases (29 healthy donors and 58 CRC cases) were randomly divided into three independent sets that were used for different purposes as follows: Set I (n=11, five healthy donor and six CRC cases); set II (n=56, 20 healthy donor and 36 CRC cases); and set III (n=20, four healthy donor and 16 CRC cases). The initial tumor stage of patients with CRC was classified using the 8th Edition of the American Joint Committee on Cancer (AJCC) staging system (38), and the healthy donor controls were requested to undergo a colonoscopy examination. All patients enrolled in the present study were managed according to standard guidelines, with regular follow-up. To characterize the targets of interest in CRC samples, the proteins encoded by these genes were immunostained in colon cancer tissue arrays (COC1021; total 102 available cores, including two non-CRC colonic tissues, one congenital megacolon, two colon adenomas, four papillary adenocarcinomas, seven mucinous adenocarcinomas, and 86 colon adenocarcinomas; Pantomics, Inc.). According to the provided TNM classification from Pantomics, Inc., 22 tissue cores with adenocarcinoma were diagnosed as AJCC stage I, 39 were AJCC stage II, and 36 were AJCC stage III (Fig. 1) (39). To validate a predictive model, other stool specimens and one cDNA array of colonic tissues were used. Briefly, stool specimens of two female patients (59 and 71 years of age) during pre- and post-surgical treatments at the Sijhih Cathay General Hospital (New Taipei City, Taiwan) and 119 individuals (age range, 26-76 years; 67 males and 52 females) following a colonoscopy at Cathay Healthcare Management (Taipei City, Taiwan) were collected. In addition, one cDNA array (HCRT104; OriGene Technologies, Inc.) of 48 colon tissues covering non-CRC status (n=8) and four CRC stages (n=40) was purchased to examine the predictive model.

LOOCV, discriminant analyses and predictive model for CRC risk. The stool specimens from set I were initially applied to screen out genes with a differential expression in previous microarray hybridizations performed by the authors (14,40). A LOOCV analysis was then performed for the additional screened genes on a given set (set II) as a learning system. Briefly, each stool specimen was excluded in turn and classified using a defined model based on the non-excluded samples (41,42). Using the cases in set II as the well-defined group, significant LOOCV-estimated molecular panels were used to test the probabilities of group membership (healthy donor or CRC) in the testing sets (sets I and III) via discriminant analysis. As a result, a predictive model of an optimal molecular panel for CRC risk was produced from all 87 well-known cases (sets I, II, and III) via the analysis of the logistic regression model (43,44), and a receiver operating characteristic (ROC) curve was generated to assess model discrimination (45).

Relative gene expression quantification. The method of purifying total RNA from stool mud [0.5 g stool in 1 ml guanidinium thiocyanate buffer; 10 mM Tris (pH 7.4), 200 mM NaCl, 1 mM EDTA (pH 8.0), 4 M guanidinium thiocyanate, and 1% β-mercaptoethanol] was largely described in previous reports by the authors (40,46). An appropriate supernatant was then extracted using a MagCore Nucleic Acid Extract kit in a MagCore Nucleic Acid Extractor (RBC Bioscience Corp.). The eluted fecal total RNA was then quantified using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Inc.) and reverse-transcribed to generate single-stranded cDNAs

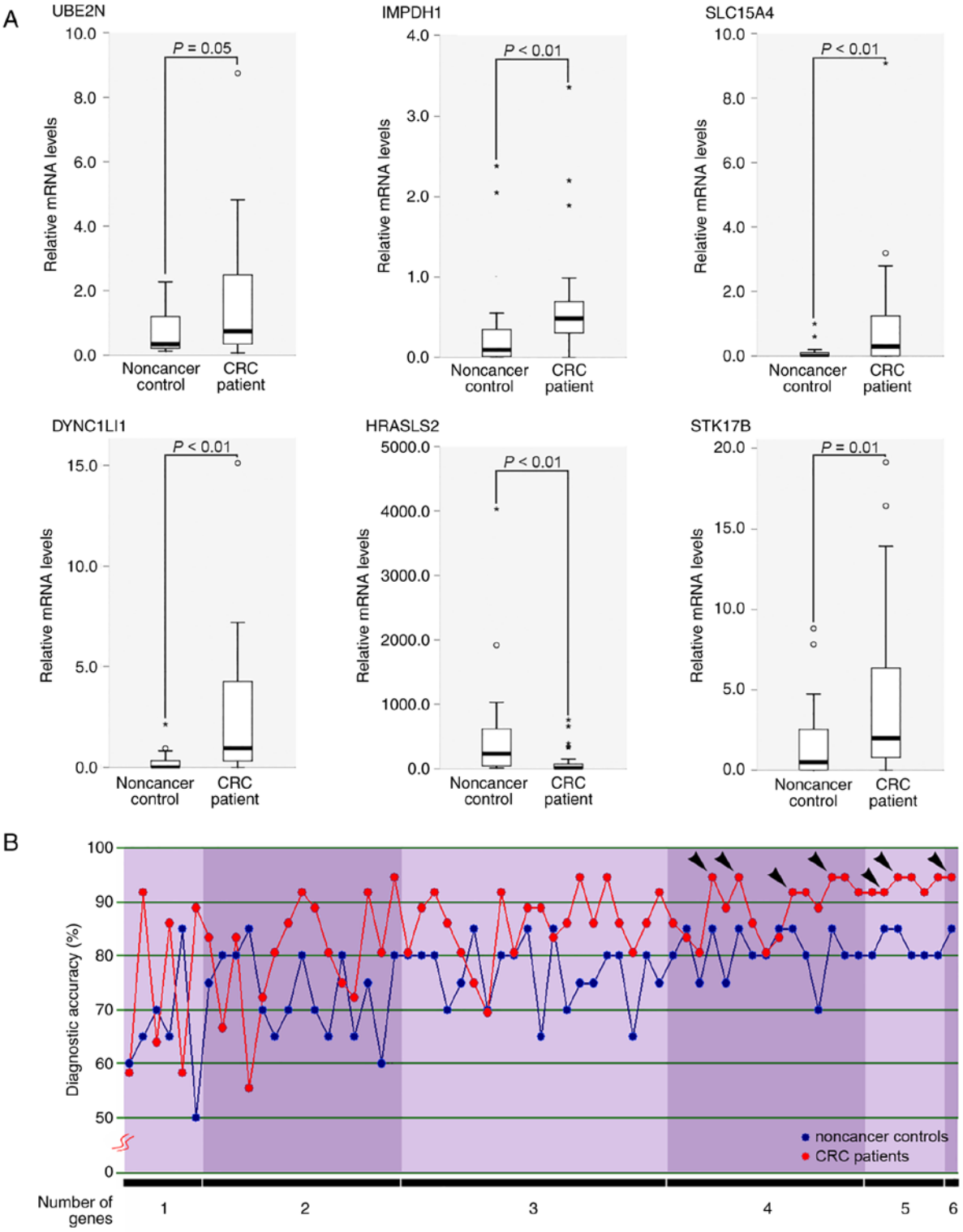

Figure 1. Development of gene panels in stool specimens for patients with CRC. (A) Statistical comparison of gene expressions between healthy donor control and CRC patients. Six genes were differentially expressed in healthy donor controls (n=20) and CRC patients (n=36). Differences in the relative levels of the target mRNAs among the samples were determined using the Mann-Whitney U test. P≤0.05 (B) Diagnostic accuracy of 63 different molecular panels. The 63 different molecular panels were comprised of one to six genes. Each panel was used to predict the healthy donor or CRC disease status using LOOCV analysis. The six genes used were UBE2N, IMPDH1, SLC15A4, DYNC1LI1, HRASLS2 and STK17B. Black arrowheads indicate the panels with higher sensitivity (≥90%) and specificity (≥85%). CRC, colorectal cancer; LOOCV, leave-one-out cross-validation; UBE2N, ubiquitin-conjugating enzyme E2 N; IMPDH1, inosine monophosphate dehydrogenase 1; SLC15A4, phospholipase A and acyltransferase 2 solute carrier family 15 member 4; DYNC1LI1, dynein cytoplasmic 1 light intermediate chain 1; HRASLS2, phospholipase A and acyltransferase 2; STK17B, serine/threonine kinase 17b.

using random primers and a PowerScript Reverse Transcriptase Kit (cat. no. RR037B; Takara Bio USA, Inc.), according to the manufacturer's instructions.

The genes of interest were quantified by a program (10 min at 95˚C, proceeding with 60 cycles at 95˚C for 10 sec and at 60˚C for 20 sec) in the presence of a TaqMan probe and

Table I. RT-qPCR primers and probe numbers for gene expression quantification.

Gene name	Accession no.	Sequence (from 5' to 3')	UPL no.
GAPDH	NM_002046	Fw: CTCTGCTCCTCCTGTTCGAC Rv: ACGACCAAATCCGTTGACTC	#60
UBE2N	NM_003348.3	Fw: AAGCCCAAGCCATAGAAACA Rv: ATGCAAACAAAGAGGAGGAAGT	#2
AKIRIN1	NM_024595.1	Fw: ACTCCTCAGCACTCACAGCA Rv: CCAACTTGTCGGAGGGTAAA	#80
IMPDH1	NM_000883.3	Fw: GTCCATGGCCTGCACTCT Rv: GTGGACACTGGGGTGCAT	#22
SLC15A4	NM_145648.3	Fw: GAGCAGTCACACAGACTTTGGT Rv: CAGGAGGGTAGCTCCTTGAA	#71
DYNC1LI1	NM_016141.2	Fw: CTGGTGTGAGTGGTGGTAGC Rv: TCTGCATGAACATCTAAGACAGG	#10
HRASLS2	NM_017878.1	Fw: ATCTGCGCTATGGCGTCT Rv: CAGCAGGATCCCCACAAG	#74
APOA1	NM_000039.1	Fw: CCTTGGGAAAACAGCTAAACC Rv: CCAGAACTCCTGGGTCACA	#39
STK17B	NM_004226.2	Fw: GGAAATCATGGGAACACCAG Rv: TTGCTGTGGTAATGGGATCAT	#50

UPL, Roche universal probe library; UBE2N, ubiquitin-conjugating enzyme E2N; AKIRIN1, akirin 1; IMPDH1, inosine monophosphate dehydrogenase 1; SLC15A4, solute carrier family 15, member 4; DYNC1LI1, dynein, cytoplasmic 1 light intermediate chain 1; HRASLS2, phospholipase A and acyltransferase 2; APOA1, apolipoprotein A-I; STK17B, serine/threonine kinase 17b.

TaqMan Master Mix using a Roche LightCycler nano system (Roche Diagnostics GmbH) according to the manufacturer's instructions. Briefly, all genes were quantified relative to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each quantification. All primers and probes used in the present study are listed in Table I. LightCycler Software (version 4.05; Roche Diagnostics GmbH) was used to analyze the PCR kinetics. Expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (47) and normalized to the expression level of GAPDH (48). Each run also included an appropriate and predetermined diluted human reference cDNA (Takara Bio USA, Inc.), which was used as a standard to estimate relative expression levels.

Immunohistochemistry. COC1021 tissue arrays (Pantomics, Inc.), which have defined clinical diagnosis and clinicopathological information, were used for immunohistochemical staining (49). Firstly, tissue arrays with 4 μm core thickness were deparaffinated and hydrated using routine protocols. Antigen retrieval was performed by steaming in Tris-EDTA buffer (pH 9.0) for 20 min, and arrays were then blocked in 1.5% (v/v) Normal Horse Serum Blocking Solution (Vector Laboratories, Inc.) for 2 h at room temperature. The specific target protein was immunodetected using an adequate primary antibody concentration [ubiquitin-conjugating enzyme E2 N (UBE2N): anti-Ube2N, cat. no. ab117090, dilution 1:20, Abcam; inosine monophosphate dehydrogenase 1 (IMPDH1): anti-IMPDH1, cat. no. ab84957, 1:20, Abcam; dynein cytoplasmic 1 light intermediate chain 1 (DYNC1LI1): anti-DLC-A, cat. no. ab154251, 1:20, Abcam; phospholipase A and acyltransferase 2 (HRASLS2): anti-HRASLS2, cat. no. bs-6013R, 1:1,000, Thermo Fisher Scientific, Inc.] in the blocking solution mentioned above at 4˚C overnight. Endogenous peroxidases in tissue sections were removed by incubation with 0.3% H_2O_2 for 15 min, and these peroxidase-free arrays were further incubated with a biotinylated secondary antibody (either goat anti-rabbit, cat. no. BA-1000-1.5, 1:200, Vector Laboratories, Inc. or rabbit anti-goat immunoglobulin G, cat. no. BA-5000-1.5, 1:200, Vector Laboratories, Inc.) at room temperature for 60 min. The VECTASTAIN ABC system and DAB Substrate kit (both from Vector Laboratories, Inc.) were used to develop the secondary antibodies captured on arrays, according to the manufacturer's instructions. Following hematoxylin (GHS3; 50 ml, Merck KGaA) counterstaining at room temperature for 5 min and slide mounting with Malinol (Muto Pure Chemicals, Co., Ltd.), images were digitalized using a high-resolution scanner (Mirax Scan, Carl Zeiss AG) at the Taiwan Mouse Clinic (Academia Sinica, Taipei City, Taiwan). Two independent pathologists reviewed and evaluated the imaging results. QuPath (Version 0.3.0; https://qupath.github.io) was employed to produce the cell densities of immunoreactive cells per mm^2 for all target proteins (50).

Statistical analyses. Differences in the relative levels of the target mRNAs among the various samples were determined using the unpaired non-parametric two-sample Mann-Whitney U test. The equation for the probability of CRC was acquired via a logistic regression model. In addition, one-way analysis of

variance (ANOVA) was performed to compare the densities of positive signaling cells for the immunohistochemical staining of target proteins. All ANOVA analyses were followed by a Bonferroni post hoc test. These deduced probabilities and identifications based on 119 individuals after colonoscopy were compared using the chi-squared test. To assess the effectiveness of the predictive model of CRC risk, a ROC curve was plotted between the sensitivity and (1-specificity), and the total area under the ROC curve (AUC) was calculated. All statistical analyses were performed with IBM SPSS Statistics for Windows (Version 22.0; IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Development of gene panels for patients with CRC. All genes were selected according to different clinical statuses from two microarray hybridizations, whole-genome oligonucleotides and custom-made microarrays with ~4,000 genes from previous studies by the authors (14,40,51). Three independent sets of stool specimens (sets I, II and III) were then used to validate gene expression by using a series of RT-qPCR assays in order to select the optimal stool-based panels to predict CRC probability. From sample set I, eight differentially expressed genes [UBE2N, akirin 1 (AKIRIN1), IMPDH1, solute carrier family 15 member 4 (SLC15A4), DYNC1LI1, HRASLS2, apolipoprotein A1 (APOA1) and serine/threonine kinase 17b (STK17B)] (P<0.2; Mann-Whitney U test) were in accordance with the corresponding expression trends obtained from the custom-made microarray hybridization (Table SI). However, six genes only (UBE2N, IMPDH1, SLC15A4, DYNC1LI1, HRASLS2 and STK17B) were differentially expressed in sample set II, which was used as the training set with statistical significance (P≤0.05; Mann-Whitney U test) (Fig. 1A). These six genes comprised 63 different molecular panels. Moreover, using the same samples of set II, each panel was used to predict the healthy donor or CRC disease status using LOOCV analysis. As demonstrated in Fig. 1B, the diagnostic accuracy of each panel for healthy donor controls or patients with CRC was ≥50%, and seven different panels (UBE2N, IMPDH1, DYNC1LI1 and HRASLS2 genes; UBE2N, IMPDH1, HRASLS2 and STK17B genes; UBE2N, DYNC1LI1, HRASLS2 and STK17B genes; IMPDH1, SLC15A4, HRASLS2 and STK17B genes; UBE2N, IMPDH1, SLC15A4, DYNC1LI1 and STK17B genes; UBE2N, IMPDH1, SLC15A4, HRASLS2 and STK17B genes; UBE2N, IMPDH1, SLC15A4, DYNC1LI1, HRASLS2 and STK17B genes) yielded higher sensitivity (≥90%) and specificity (≥85%). A four-gene panel composed of only three upregulated genes (UBE2N, IMPDH1 and DYNC1LI1) and one downregulated (HRASLS2) in patients with CRC exhibited the highest accuracy for healthy donor controls (88.9%, eight of nine) and patients with CRC (95.4%, 21 of 22) in predicting the testing sets (sets I and III) and using sample set II as the training set, as assessed by discriminant analyses.

Relative protein expression levels of the four-gene panel in CRC tissues. Subsequently, it was investigated whether the corresponding proteins of four genes were differentially expressed in tumor samples from patients with CRC. An array method based on established immunohistochemistry protocols was used to detect the corresponding protein expression in human colonic tissues (Fig. 2 for overview of COC1021 tissue array with 102 cores (x-axis, nos. 1 to 13; y-axis, a to h); Fig. 3 for immunohistochemical staining of HRASLS2; Fig. 4 for immunohistochemical staining of UBE2N; Fig. 5 for immunohistochemical staining of IMPDH1; Fig. 6 for immunohistochemical staining of DYNC1LI1). Moreover, CRC tissues at different stages with CRC and non-CRC fractions in the same donor of the COC1021 tissue array revealed that the signal density (positive cells/mm^2) of UBE2N (Fig. 4B: 2,139 cells/mm^2 in c12, 2,802 cells/mm^2 in e7, and 3,156 cells/mm^2 in g9), IMPDH1 (Fig. 5B: 1,729 cells/mm^2 in c12, 732 cells/mm^2 in e7, and 2,234 cells/mm^2 in g9), or DYNC1LI1 (Fig. 6B: 3,905 cells/mm^2 in c12, 2,272 cells/mm^2 in e7, and 2,061 cells/mm^2 in g9) was high in the CRC fractions and low in the adjacent non-CRC fractions (Fig. 4B: 1,050 cells/mm^2 in c12, 1,411 cells/mm^2 in e7, and 556 cells/mm^2 in g9; Fig. 5B: 452 cells/mm^2 in c12, 625 cells/mm^2 in e7, and 1,011 cells/mm^2 in g9; Fig. 6B: 1,523 cells/mm^2 in c12, 1,814 cells/mm^2 in e7, and 785 cells/mm^2 in g9). By contrast, HRASLS2 was expressed with a high density of positive cells in the non-CRC fractions (1,993 cells/mm^2 in c12, 3,538 cells/mm^2 in e7, and 545 cells/mm^2 in g9), but relatively less densely expressed in the adjacent CRC fractions (1,228 cells/mm^2 in c12, 3,153 cells/mm^2 in e7, and 403 cells/mm^2 in g9) (Fig. 3B).

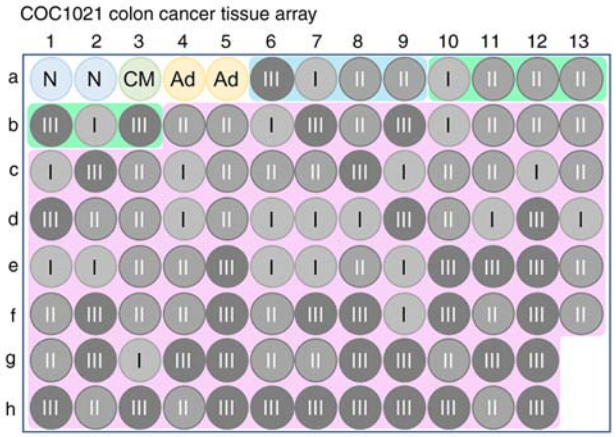

Figure 2. COC1021 colon cancer tissue array. A total number of 102 available cores was enrolled (102 cores: x-axis, nos. 1 to 13; y-axis, a to h). N, non-CRC colonic tissue; CM, congenital megacolon; Ad, colon adenoma; I, AJCC stage I; II, AJCC stage II; III, AJCC stage III. Blue area, papillary adenocarcinoma; green area, mucinous adenocarcinoma; pink area, colon adenocarcinoma. CRC, colorectal cancer; AJCC, American Joint Committee on Cancer.

A logistic regression model to predict CRC disease. Three sets of stool specimens (n=87) were then pooled to produce a binary logistic regression equation as follows: $Prob_{CRC}=1/1 + e^{-z}$, where $Prob_{CRC}$ is the probability of CRC disease, 'e' denotes the exponential function, and 'z' is equal to 5.468 + 1.394 x log(UBE2N) + 0.859 x log(IMPDH1) + 1.129 x log(DYNC1LI1)-1.471 x log(HRASLS2). The overall accuracy of this model to predict CRC in the participants was 94.3%. The sensitivity was 96.6% (56 out of 58), and the specificity

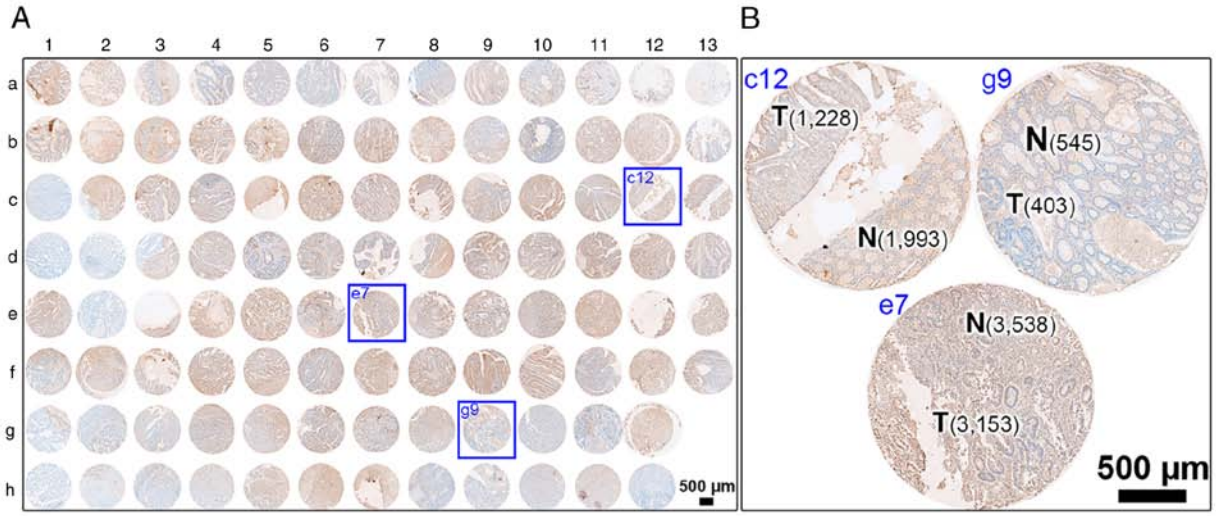

Figure 3. Immunohistochemical staining for the expression of HRASLS2 protein in the CRC tissue array. (A) Overview of a tissue array with the HRASLS2 immunoactivity. (B) Representative images of immunohistochemical staining for HRASLS2 protein. Protein signal densities of three CRC tissues (c12 and e7 at AJCC stage I; g9 at AJCC stage III) are shown as immunoreactive cells per mm^2 and displayed in parentheses for the CRC fraction (T) and the adjacent non-CRC fraction (N). There were 102 cores (x-axis, nos. 1 to 13; y-axis, a to h). The scale bar corresponds to 500 μm. HRASLS2, phospholipase A and acyltransferase 2; CRC, colorectal cancer.

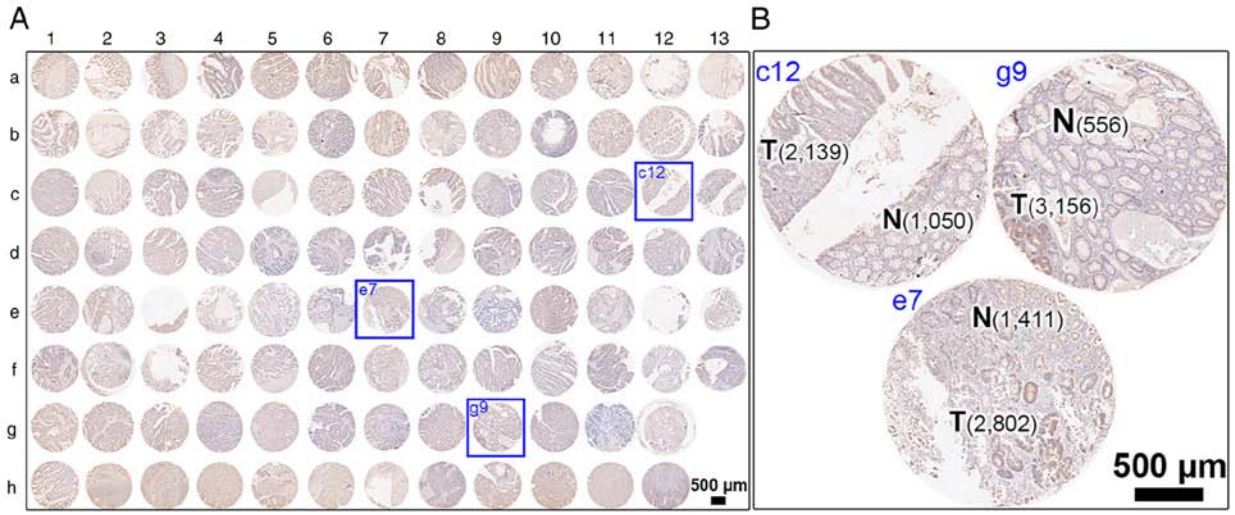

Figure 4. Immunohistochemical staining for the expression of UBE2N protein in the CRC tissue array. (A) Overview of a tissue array with the UBE2N immunoactivity. (B) Representative images of immunohistochemical staining for UBE2N protein. Protein signal densities of three CRC tissues (c12 and e7 at AJCC stage I; g9 at AJCC stage III) are shown as immunoreactive cells per mm^2 and displayed in parentheses for the CRC fraction (T) and the adjacent non-CRC fraction (N). There were 102 cores (x-axis, nos. 1 to 13; y-axis, a to h). The scale bar corresponds to 500 μm. UBE2N, ubiquitin-conjugating enzyme E2 N; CRC, colorectal cancer; AJCC, American Joint Committee on Cancer.

was 89.7% (26 out of 29). The positive predictive value (PPV) was 95.1% (56 out of 59), and the negative predictive value (NPV) was 93.9% (26 out of 28). Thus, this logistic regression model was used for the prediction of increased risk for CRC in patients, among new study participants. Furthermore, the AUC for the assessment of the model discrimination was 0.957 [95% confidence interval (CI), 0.914-1.000; P<0.01] (Fig. 7). The cut-off value for predicting CRC ($Prob_{CRC}$) was 0.540, with 96.6% (95% CI, 88.1-99.6%) sensitivity and 89.7% (95% CI, 72.6-97.8%) specificity (Table II).

Model validity of patients with CRC and healthy individuals. First, pre- and post-surgical stool specimens were obtained from two patients with CRC (pCCG1: AJCC stage III; pCCG2: AJCC stage II). In addition, stool specimens were obtained from another patient with CRC (a 49-year-old female) as a positive CRC control and a healthy donor individual (a 72-year-old male) with negative colonoscopy results as a negative control. As demonstrated in Fig. 8A, the highest positive predicted values for CRC disease were detected for the two pre-surgical samples (pCCG1, 0.905; pCCG2, 1.000) as compared with the healthy donor control (0.199), as assessed using the logistic regression model of the four-gene panel. By contrast, markedly lower predicted values were detected in post-surgical samples (0.093 for pCCG1; 0.284 for pCCG2), which were prepared from the feces of two patients whose tumors were completely

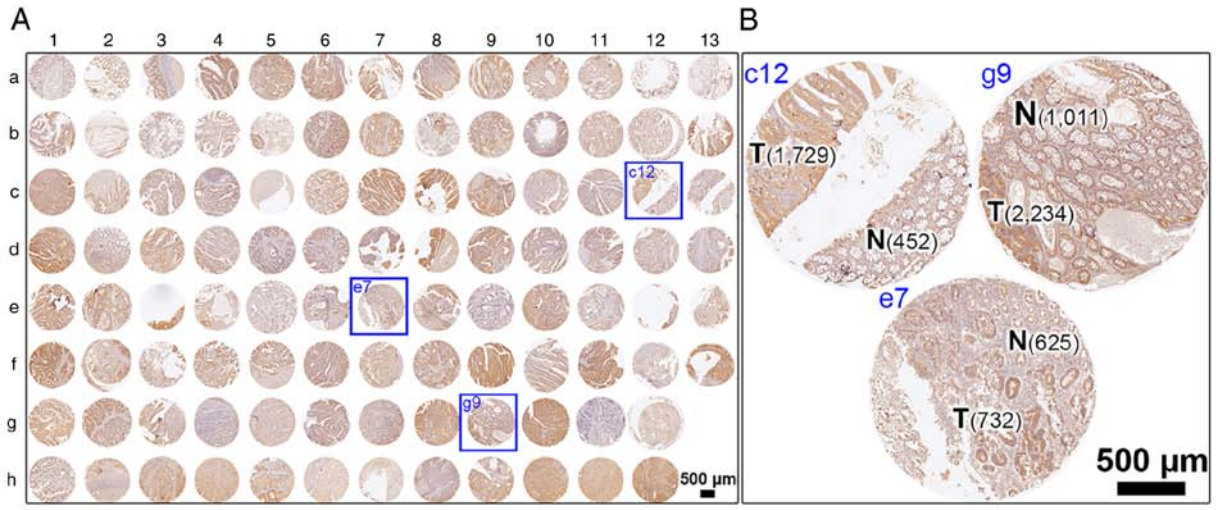

Figure 5. Immunohistochemical staining for the expression of IMPDH1 protein in the CRC tissue array. (A) Overview of a tissue array with the IMPDH1 immunoactivity. (B) Representative images of immunohistochemical staining for IMPDH1 protein. Protein signal densities of three CRC tissues (c12 and e7 at AJCC stage I; g9 at AJCC stage III) are shown as immunoreactive cells per mm^2 and displayed in parentheses for the CRC fraction (T) and the adjacent non-CRC fraction (N). There were 102 cores (x-axis, nos. 1 to 13; y-axis, a to h). The scale bar corresponds to 500 μm. IMPDH1, inosine monophosphate dehydrogenase 1; CRC, colorectal cancer; AJCC, American Joint Committee on Cancer.

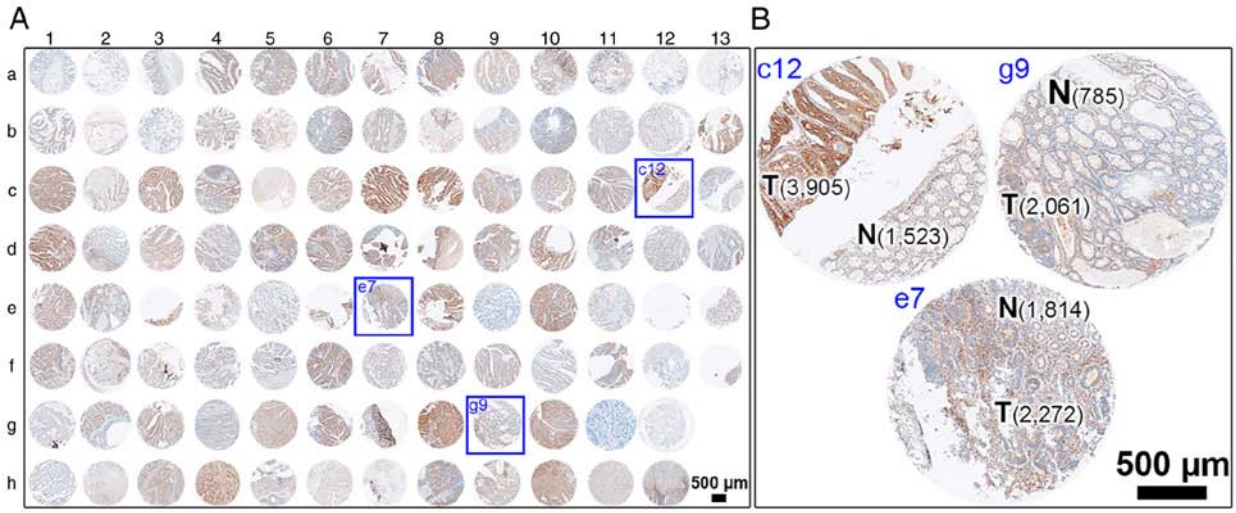

Figure 6. Immunohistochemical staining for the expression of DYNC1LI1 protein in the CRC tissue array. (A) Overview of a tissue array with the DYNC1LI1 immunoactivity. (B) Representative images of immunohistochemical staining for DYNC1LI1 protein. Protein signal densities of three CRC tissues (c12 and e7 at AJCC stage I; g9 at AJCC stage III) are shown as immunoreactive cells per mm^2 and displayed in parentheses for the CRC fraction (T) and the adjacent non-CRC fraction (N). There were 102 cores (x-axis, nos. 1 to 13; y-axis, a to h). The scale bar corresponds to 500 μm. DYNC1LI1, dynein cytoplasmic 1 light intermediate chain 1; CRC, colorectal cancer; AJCC, American Joint Committee on Cancer.

removed. It is notable that the timepoint of collection of the postsurgical samples was at least 1 month after surgery, with patient pCCG1 receiving further chemotherapy.

This specific four-gene panel was then used to test the 119 stool specimens that were obtained before colonoscopy examination. Briefly, 112 cases presented with various hemorrhoids, 51 with polyps, eight with colitis, four with CRC, and only one was entirely healthy. Among the 43 cases (36.1%, 43 out of 119) with a higher ProbCRC (>0.540), four were proven to have CRC (mean ProbCRC=0.968), five had colitis (mean ProbCRC=0.991) and 16 had polyps (mean ProbCRC=0.834) following a colonoscopy examination (Table III). Conversely, up to 62.5% (5 out of 8) of the cases with colitis or proctitis were predicted to be positive, with only 31.4% (16 out of 51) of the cases with polyps exhibiting increased predictive rates.

Finally, the ProbCRC of each case was calculated in the HCRT104 cDNA array composed of 48 colonic tissues. The mean ProbCRC of 40 patients with CRC was 0.913 (range: 0.535-0.997), which was significantly higher than that of eight non-CRC colon tissues (0.459; range: 0.042-0.740) (Fig. 8B). In particular, the mean ProbCRC of 10 patients with CRC with distant metastasis was up to 0.973 (range, 0.924-0.997) (Table SII). Using this predictive model, 39 patients with CRC were identified as positive cases (ProbCRC >0.540), and the PPV was up to 97.5% (39 of 40). In addition, five non-CRC cases were correctly diagnosed as negative cases (ProbCRC ≤0.540), with the corresponding NPV being 62.5% (5 of 8) (Table SIII).

Table II. Sensitivity and specificity for predicting colorectal cancer.

Criterion	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Accuracy (%)
0.006	98.3	90.8-100.0	6.9	0.8-22.8	69.2
0.264	98.3	90.8-100.0	75.9	56.5-89.7	90.1
0.540	96.6	88.1-99.6	89.7	72.6-97.8	94.5
0.714	82.8	70.6-91.4	93.1	77.2-99.2	86.8
0.950	62.1	48.4-74.5	96.6	82.2-99.9	74.7

CI, confidence interval.

Table III. Prediction of CRC by the four-gene panel for the 119 patients with CRC.

Features	Case no.	Probability of CRC (%) >0.540 (positive no.)	P-value
Age, years			
<49	57	36.8% (21)	0.878
≥49	62	35.5% (22)	
Sex			
Male	65	41.5% (27)	0.178
Female	54	29.6% (16)	
CRC			
Negative	115	33.9% (39)	0.016
Positive	4	100.0% (4)	
Colitis/proctitis			
Negative	107	31.8% (34)	0.077
Positive	8	62.5% (5)	
Polyp			
Negative	64	35.9% (23)	0.607
Positive	51	31.4% (16)	
Hemorrhoid			
Negative	3	33.3% (1)	0.265
Internal	94	37.2% (35)	
External	2	50.0% (1)	
Mixed	16	12.5% (2)	

CRC, colorectal cancer.

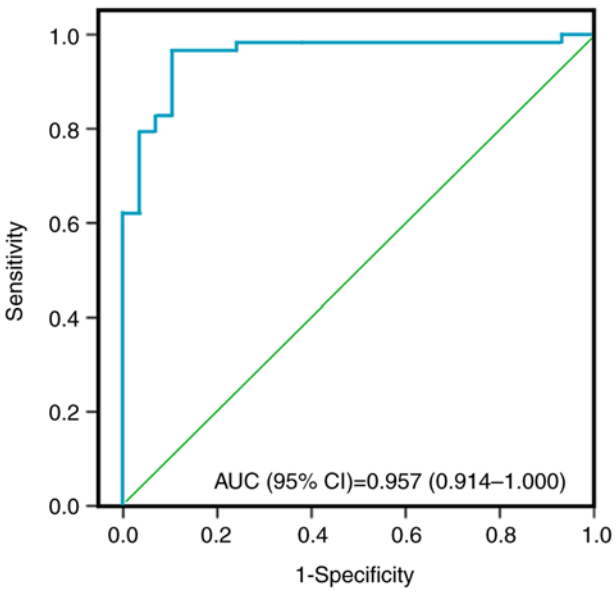

Figure 7. The analysis of the receiving operating characteristics curve demonstrating the ability of the predictive model (UBE2N, IMPDH1, DYNC1LI1 and HRASLS2) to discriminate the CRC group. UBE2N, ubiquitin-conjugating enzyme E2 N; IMPDH1, inosine monophosphate dehydrogenase 1; DYNC1LI1, dynein cytoplasmic 1 light intermediate chain 1; STK17B, serine/threonine kinase 17b; HRASLS2, phospholipase A and acyltransferase 2; CRC, colorectal cancer.

Discussion

The early detection of CRC would permit timely surgical intervention in patients and this in turn would halt tumor progression, thus enabling effective therapy (52,53). Sigmoidoscopy and colonoscopy are simultaneously used at present for the early detection of CRC. However, these are invasive techniques (54-56). By contrast, clinical examinations are limited due to their risk and inconvenience. Consequently, a non-invasive fecal occult blood test is currently and widely applied for CRC screening, even though it has poor specificity and sensitivity (57).

CRC is believed to develop slowly via the progressive accumulation of genetic mutations (58,59). Genes involved in human diseases, tumorigenesis, or even those with unrecognized functions are potential markers that may aid in the diagnosis of CRC. A variety of molecular approaches are emerging to improve the effectiveness and user-friendliness of non-invasive CRC screening (60,61). In the present study, it was demonstrated that limited genetic markers rarely discussed in CRC were able to screen or detect CRC. In other words, it is not necessary to examine additional genes in the field of clinical practice for CRC when the sample type used for examination is stool instead of blood (61,62). For example, assays that aim to determine early alterations in gene expression in stool specimens due to malignancy, appear to be a promising alternative to the detection methods currently used (23,63). In fact, stool specimens have been used as a representative of CRC manifestations for a number of years (30,64,65). Either CRC tissues or human stool specimens can serve as an appropriate object to explore CRC

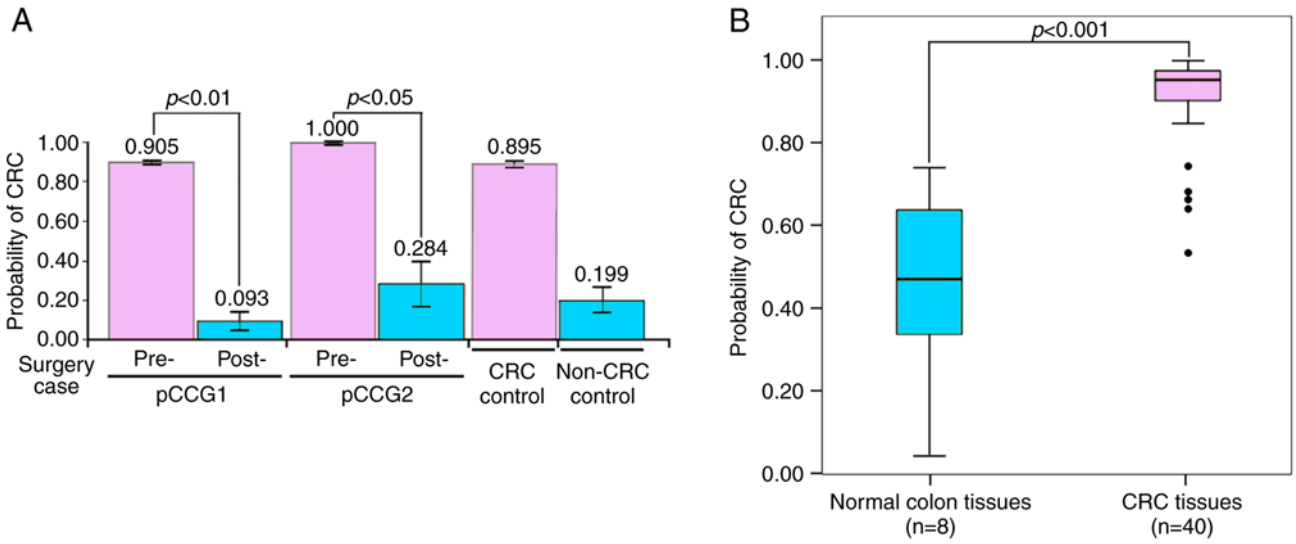

Figure 8. Model validity of patients with CRC and non-CRC controls. (A) Probability of CRC from pre- and postsurgical stool specimens. Data are presented as the mean ± standard error of the mean (n=2). (B) Boxplot showing the probability of CRC cDNA array. HCRT104 cDNA array (OriGene Technologies, Inc.) of 48 colon tissues was used. Data were presented as the mean ± standard error of the mean of non-CRC status (n=8) and four CRC stages (n=40). Black spot, outliers. CRC, colorectal cancer.

development (27,28). Genetic information from these two clinical materials is significantly interrelated (56,66). This is also reflected in the results of the present study, where the differentially expressed genes detected in the stool specimens of patients with CRC were also detected at the mRNA and protein levels, in CRC tissues. These findings suggest that a high gene expression in stool specimens may indicate the presence of colorectal neoplasia in the colonic tract and may provide an instrument for understanding CRC development (67-69). Moreover, this non-invasive approach can greatly enhance screening acceptance and be implemented in public health services due to its relative ease of use and cost-effectiveness (52).

Specific gene expression profiles have been used to classify tumors or predict prognoses (70,71). In CRC research, whole-genome analyses of human stool specimens have been performed by several groups (14,72-74). Miyake *et al* (2) revealed that a discriminator gene set could predict metastases from tissues of patients with early-stage CRC. As an example of translation from basic science to clinical care, these results suggested that understanding the molecular mechanisms of CRC tumorigenesis may lead to new approaches for the diagnosis of CRC. The results of the present study provide evidence to support this concept (75). Actually, eight differentially expressed genes (UBE2N, AKIRIN1, IMPDH, SLC15A4, DYNC1LI1, HRASLS2, APOA1 and STK17B) were originally selected. However, six genes (UBE2N, IMPDH, SLC15A4, DYNC1LI1, HRASLS2 and STK17B) were used for the final LOOCV analysis in the present study, after validating all corresponding relative expression using a series of RT-qPCR assays for sample set II. Finally, only four genes (UBE2N, IMPDH1, DYNC1LI1 and HRASLS2) presented with high sensitivity (96.6%) and high sensitivity (89.7%). As a result, it was ultimately decided to include only four, rather than eight or six genes, for subsequent experiments in the present study. Stool-based assays were used to detect CRC and proposed this specific gene panel (four-gene panel: UBE2N, IMPDH1, DYNC1LI1 and HRASLS2) that exhibited the best predictive power. Furthermore, apart from achieving a high sensitivity for the detection of CRC in patients using this four-gene panel, it also identified cases with a high risk for CRC, including cases presenting with inflammation (e.g., colitis). This finding may indicate that individuals with colitis are at an increased risk of developing CRC (76). In addition, the panel used in the present study may be applied to disease tracking and treatment strategies due to the reduced positive predicted values obtained for the post-surgical stool specimens. This implies that the results of the present study may be used for the evaluation and selection of the best treatment option for individual patients and may allow for more intensive follow-up examinations for post-surgical or post-chemotherapeutic patients with CRC.

Varying levels of gene expression in stool specimens were reflected at the respective protein and mRNA levels in the tissues using immunohistochemistry and the CRC cDNA array, respectively. As previously reported by the authors, DYNC1LI1 was expressed at a significantly higher level in cDNA samples from patients with distant metastasis than in cDNA samples from non-metastatic patients and in normal colonic tissues (77). In addition, increased (UBE2N and IMPDH1) and decreased (HRASLS2) cDNA levels were observed to be statistically significant for CRC tissues of all stages (P=0.020 for UBE2N, P=0.032 for IMPDH1 and P<0.001 for HRASLS2) compared with those detected in eight cDNA samples of non-CRC tissues in the HCRT104 cDNA array (Table SIV). The densities of positive signaling cells for HRASLS2 (Fig. S1A), UBE2N (Fig. S1B), IMPDH1 (Fig. S1C), and DYNC1LI1 (Fig. S1D) varied with the grade of differentiation. Briefly, poorly differentiated (G3) CRC had relatively low HRASLS2- and high UBE2N-expressing cell densities, compared to that of normal/benign tissues or well-differentiated (G1) and moderately differentiated (G2) CRCs with statistical

significance (P<0.05 for HRASLS2, G2 vs. G3; P<0.0001 for UNE2N, G1 vs. G3 and G2 vs. G3). Although there was no statistically significant difference in the expression of IMPDH1 and DYNC1LI1 among the groups, their expression levels in differently differentiated CRC tissues still exhibited a tendency to increase gradually with a poorer differentiation. However, further analyses using a greater number of samples are required to confirm these results. The product of UBE2N, also known as UBC13, is a member of the E2 ubiquitin-conjugating enzyme family (78). These ubiquitin ligase components may function as oncogenes in several malignancies, including CRC (79,80). This may also apply to diffuse large B-cell lymphoma, in which the reduced expression of UBE2N inhibits tumor cell survival (81). Accordingly, it was inferred that this aberrant expression in CRC may upregulate the UBC13-p53 complex, which may impede the normal function of the p53 tumor suppressor (82,83). The second upregulated gene, IMPDH1, is also a crucial factor in p53-dependent growth regulation (84). IMPDH1 upregulation is tightly associated with proliferative phenotypes, including malignancy (85). Taken together with the results of the present study, it may be suggested that the increased expression of IMPDH1 in colonic cells is closely related to the occurrence of CRC. Moreover, the overexpression of the protein products of UBE2N and IMPDH1 in the mucosa and submucosa of the colonic tract were also observed. This may imply that the invasion of CRC cells into the inner cellular layers can also be encountered in stool specimens. The results of the present study may provide evidence that human stool specimens are suitable for CRC detection (14,86). In addition to the genes that were upregulated, HRASLS2, which is the only gene that was downregulated in this four-gene panel, was reported to suppress the growth of cancer cells and might be a tumor suppressor (87,88). Nevertheless, the present study is the first report, to the best of our knowledge, discussing the association between the downregulation of HRASLS2 and CRC. Moreover, the loss of repressing RAS activities may lead to CRC tumorigenesis, as HRASLS2 was downregulated in colonic cells (89).

It is hoped that new insights into colorectal carcinogenesis and personalized prediction can be achieved in a non-invasive manner. One of the key elements in CRC therapy is the ability to detect the disease easily, including using non-invasive screening methods (90,91). The results of the present study suggest that stool specimens may faithfully reproduce the actual health status of the colonic tract. Using this four-gene panel, it was demonstrated that CRC screening or detection in stool specimens can be performed without the inclusion of an excessive number of genes and that this type of sample, which is collected non-invasively, being also able to reflect colonic defects via the determination of aberrant protein expression in the colonic mucosa or submucosa. It is possible to evaluate chemotherapeutic efficacy or further elucidate the selection of the optimal treatment option from stool specimens of individual cases, anytime and anywhere. However, since RNA quality is critical for obtaining strong prognostic and predictive values, fresh stool specimens or stool in qualified storage buffers are of utmost importance and a limitation of this assay. By contrast, to improve the accuracy of this analysis, further studies are necessary to expand the sample size of training set.

In conclusion, this specific four-gene panel, consisting of UBE2N, IMPDH1, DYNC1LI1 and HRASLS2, represents a clinical tool as potential prognostic and predictive targets in stool specimens for CRC. The overexpression of UBE2N, IMPDH1 and DYNC1LI1, and the downregulation of HRASLS2 was also detected in CRC tissues. This molecular panel can be non-invasively and easily detected from stool specimens and may allow for the assessment of CRC screening, treatments, and follow-ups. The present study comprehensively demonstrates the translation of basic science into clinical practice.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The conceptualization of the present study was performed by CCH and YNC. CYS, CYL, MHS and CJH curated all data analyzed and used in the present study. SCC, WCK and CJH performed the gene quantifications. SLG performed the statistical analysis. CCH, CYL and CJH performed and analyzed the pathological data from the experiments. CCH and SCC acquired resources. CJH supervised the study. CYS, CYL and MHS drafted the original manuscript. CCH, YNC, CYS and CJH reviewed and edited the manuscript. All authors have read and approved the final manuscript. CCH and CJH confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Cathay General Hospital, Taiwan, R.O.C. (approval no. CGH-P101014). All the participating subjects provided their written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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