Gene Expression Analysis Using a Highly Sensitive DNA Microarray for Colorectal Cancer Screening

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Abstract. *Background/Aim: Half of all patients with small, right-sided, non-metastatic colorectal cancer (CRC) have negative results for the fecal occult blood test (FOBT). In the present study, the usefulness of CRC screening with a highly sensitive DNA microarray was evaluated in comparison with that by FOBT using fecal samples. Materials and Methods: A total of 53 patients with CRC and 61 healthy controls were divided into "training" and "validation sets". For the gene profiling, total RNA extracted from 0.5 g of feces was hybridized to a highly sensitive DNA chip. Results: The expressions of 43 genes were significantly higher in the patients with CRC than in healthy controls ($p<0.05$). In the training set, the sensitivity and specificity of the DNA chip assay using six genes were 85.4% and 85.2%, respectively. On the other hand, in the validation set, the sensitivity and specificity of the DNA chip assay were 85.2% and 85.7%, respectively. The sensitivities of the DNA chip assay were higher than those of FOBT in cases of the small, right-sided, early-CRC, tumor invading up to the muscularis propria (i.e. surface tumor) subgroups. In particular, the sensitivities of the DNA chip assay in the surface tumor and early-CRC subgroups were significantly higher than those of FOBT ($p=0.023$ and 0.019, respectively.). Conclusion: Gene profiling assay using a highly sensitive DNA chip was more effective than FOBT at detecting patients with small, right-sided, surface tumor, and early-stage CRC.*

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Colorectal cancer (CRC) is one of the most frequently encountered malignancies in the world (1, 2). A 5-year survival rate of more than 90% can be expected in patients with CRC if the cancer is diagnosed at an early stage (1, 3). Thus, screening for early detection of CRC is known to have an important impact on reducing the mortality rate from CRC. The fecal occult blood test (FOBT) is widely used as a screening test for CRC based on reports from three randomized controlled trials that the mortality rate from CRC could be reduced by using FOBT (4-6). Some studies showed the usefulness of FOBT using total colonoscopy in all participants as the reference standard (7-10). However, these large-scale studies have also shown that the sensitivity of FOBT, is limited. One of the limitations of FOBT is that the test detects fecal hemoglobin rather than any molecule derived directly from CRC.

To resolve this issue, novel molecular methods based on fecal protein, DNA, and RNA analyses have been developed. For the case of fecal proteins, several marker proteins indicating intestinal bleeding, such as transferrin (11), calprotectin (12, 13), and calgranulin B (14), as well as cancer cell-specific proteins, such as tumor M2-PK (15, 16), have been investigated. The reported sensitivity and specificity of the fecal M2-PK test for detecting CRC are 79%-80% and 81%-95%, respectively. For the case of fecal DNA, a number of gene mutation and gene methylation analyses have been reported (9, 17, 18). Subsequently, in 2008, the American Cancer Society recommended fecal DNA analysis as a screening method for CRC (19). Although some of these tests may have better sensitivity than FOBT, no screening test based on a fecal molecular marker has yet been

Table I. *Clinicopathological characteristics of patients with colorectal cancer (CRC) and healthy controls.*

	Training set		Validation set	
	CRC patients (n=41)	Healthy controls (n=54)	CRC patients (n=12)	Healthy controls (n=7)
Median age (range) years	60 (36-75)	62 (40-76)	69 (54-78)	57 (41-73)
Gender (n, %)				
Male	25 (61.0%)	36 (66.7%)	6 (50.0%)	4 (57.1%)
Female	16 (39.0%)	18 (33.3%)	6 (50.0%)	3 (42.9%)
Tumor size (mm, median, range)	40 (10-125)		24 (15-68)	
Tumor location (n, %)				
Rt colon	6 (14.6%)		5 (41.7%)	
Lt colon	35 (85.4%)		7 (58.3%)	
Tumor depth (n, %)				
T1 and T2	16 (39.0%)		6 (50.0%)	
T3 and T4	25 (61.0%)		6 (50.0%)	
Dukes' stage (n, %)				
A and B	25 (61.0%)		8 (66.7%)	
C and D	16 (39.0%)		4 (33.3%)	

Rt colon: Tumor was located in the cecum, ascending colon or transverse colon; Lt colon: tumor was located in the descending colon, sigmoid colon or rectum; T1 and T2: tumor invading up to the muscularis propria; T3 and T4: tumor invading beyond the *muscularis propria*.

reported that can reduce the mortality rate of CRC in comparison to that by FOBT.

For the case of fecal RNA, gene expression analyses of CRC-specific genes, especially cyclooxygenase-2 (COX2) (20), and microRNAs, especially miR-106a (21-23), have been investigated over the past decade. In these methods, the background expression levels were also increased and slight changes of gene expression could not be recognized. Moreover, the conventional reverse transcription-polymerase chain reaction (RT-PCR) method cannot be used to analyze many targets at the same time. In this context, cDNA microarray is useful for multi-target analysis. However, very few fecal RNAs can be studied using a cDNA microarray because conventional cDNA microarray can miss changes in the expression of genes with very low expression levels (24). Thus, a highly sensitive DNA chip was established to resolve this issue (25, 26) and appears to be more effective for detecting changes in the expression of genes with low expression levels as compared to conventional DNA chip. In the present study, fecal samples from patients with CRC and healthy controls without any abnormal lesions were analyzed by FOBT and the highly sensitive DNA microarray.

Materials and Methods

Study participants. From December 2010 to January 2012, 41 patients with CRC and 54 healthy controls in the present training set and 12 patients with CRC and seven healthy controls were enrolled in the present validation set. The characteristics of these patients and controls are summarized in Table I. All the patients had undergone surgical resection of their primary cancer at the National Cancer Center Hospital, Tokyo, Japan, and the National Cancer Center Hospital East, Kashiwa, Japan. All the healthy controls were determined as having no evident abnormalities by screening colonoscopy performed at the Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan. All participants were provided with detailed information about the study, and each gave written consent for participance. The study protocol was approved by the Institutional Review Board of the National Cancer Center, Japan (approval number: 13-11).

Collection of fecal samples. Naturally-evacuated fecal samples were obtained from patients with CRC before they underwent surgical resection. Fecal samples were also obtained from healthy controls a few weeks after they had undergone the screening colonoscopy. All participants were instructed to evacuate at home into a disposable 5 cm × 10 cm polystyrene tray (AsOne, Osaka, Japan) and bring the sample to the outpatient clinic. The fecal samples were processed for the next step immediately after they were brought to our laboratory.

Quantitative immunochemical FOBT. Ten milligrams of each fecal sample was transferred into the fecal sample container provided with the immunochemical FOBT kit, Hemo-Plus (Alfresa Pharma, Osaka, Japan). FOBT was immediately performed using Hemo-Plus, in accordance with the manufacturer's instructions. The detection range of the fecal hemoglobin concentration for this kit was 20 ng/ml to 1,200 ng/ml. The cut-off value of the fecal hemoglobin concentration measured by the FOBT kit for a positive result was 100 ng/ml.

Extraction of total RNA from feces. In order to protect the fecal RNA from RNase degradation, 0.5 g of each fecal sample was stored in 10 ml of RNAlater® Solution (Ambion, Ausin, TX) at 4˚C. Within 2 days, total RNA from the fecal samples was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction, but with slight modification. Briefly, each fecal sample was homogenized by vortexing in 2 ml of Buffer RLT mixed with 20 μl β-mercaptoethanol for 1 min. The fecal mixture

was centrifuged at 8,000 × *g* for 5 min at room temperature, and the upper aqueous phase was transferred to a new tube and mixed with an equal volume of 70% ethanol. The mixture was processed according to the manufacturer's instructions, except that the RNeasy spin column membrane was washed four times with Buffer RPE. The quality of the total RNA was determined using a NanoDrop® ND-1000 spectrometer (ThermoFisher Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, CA, USA). The RNA samples were stored at –80˚C until subsequent analysis.

Gene expression profiling. Total RNA was amplified using the TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit 101 (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. The amplified aRNA was labeled with Cy5 dye, and hybridized for 16 h to the highly sensitive DNA microarray, 3D-Gene® Human Oligo chip 25 k, ver. 2.1 (Toray Industries, Tokyo, Japan) which permits the detection of 24,460 mRNAs. The DNA microarray was washed according to the manufacturer's instructions, followed by image scanning using 3D-Gene® Scanner 3000 (Toray Industries) and data processing using 3D-Gene® Extraction 2.0.0.4 (Toray Industries).

The mean intensity and standard deviation (SD) of the background signal were calculated using the signal intensities of the blank spots residing within the 95% confidence intervals. Genes with signals greater than 2 SD of the background signal were considered as exhibiting positive expression. The mean background level was subtracted from the signals of the detected genes.

Statistical analysis. Statistical analyses were performed using the Comprehensive R software, version 2.15.1 (available at http://cran.r-project.org/). First of all, all the samples obtained from the 53 patients with CRC and 61 healthy controls were divided into the training set of 41 patients with CRC and 54 healthy controls, and the validation set of 12 patients with CRC and seven healthy controls. The genes with signal intensities in excess of 26 in more than 20% of the patients of the training set were selected as the diagnostic marker candidates. Differences in gene expressions between the patients with CRC and healthy controls of the training set were analyzed by the Wilcoxon signed-rank test (unpaired, two-sided). The *p*-values were adjusted for multiple testing by Bonferroni's correction and calculated p-values of less than 0.05 were considered to denote statistical significance. Classification of patients with CRC and healthy controls was performed by the one-class Support Vector Machine (one-class SVM) (27-29). Briefly, radial basis function was selected as the kernel function, and the parameters were optimized by grid-search using cross-validation for the training set, and then the same parameters were applied for the validation set, according to the manufacturer's instructions. Firstly, the expression value of the gene the smallest *p*-value was applied to one-class SVM. One-class SVM was repeated using combinations of genes; the number of genes used for one-class SVM was increased one by one in ascending order of the *p*-values until the 43rd gene was included in the combination of the classifiers. For each classification output of the one-class SVM, the sensitivity, specificity and accuracy were calculated. Finally, the genes for which the maximum accuracy was obtained were considered as the biomarker candidates for the detection of CRC. The sensitivity, specificity, and accuracy were calculated using the following formulae: sensitivity=(number of true-positive patients)/(number of all patients); specificity=(number of true-negative controls)/(number of all controls); accuracy=(number of true-positive patients and true-negative controls)/(number of all patients and controls). The differences in the sensitivities of FOBT and DNA chip assay were analyzed by the χ^2 test. A value of $p<0.05$ was considered to denote statistical significance.

Results

Differentially expressed genes between patients with CRC and healthy controls. Among the 24,460 genes contained in the DNA chip 3D-Gene®, the median number of detected genes was 6,379 (26.1%) in the 53 patients with CRC and 3,047 (12.5%) in the 61 healthy controls. In the training set, 399 genes satisfied the criteria of the signal intensity exceeding 2^6 in more than 20% of the training set. Of these 399, the expressions of 43 genes were significantly higher in the patients with CRC as compared to the expression levels in the healthy controls ($p<0.05$) (Table II). Thus, these genes were considered as biomarker candidates for the detection of CRC.

Sensitivity, specificity and accuracy of the DNA chip assay and FOBT. The sensitivity, specificity and accuracy using the 43 genes with differential expressions between the patients with CRC and healthy controls were calculated by one-class SVM analysis. The best accuracy was 85.3% in the training set using 6 genes, and 84.2% in the validation set using 5 to 7 genes (Figure 1). The 6 genes of the training set, consisting of CCAAT/enhancer binding protein, beta (*CEBPB*); Fc fragment of IgG, low-affinity IIIa, receptor (*FCGR3A*); 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (*PFKFB3*); interleukin 8 (*IL8*); superoxide dismutase 2 (*SOD2*); and regulator of G-protein signaling 2 (*RGS2*) were considered as markers of CRC. The sensitivity, specificity, and accuracy of the DNA chip assay and FOBT were shown in Table III. In the training and validation sets, the accuracy for the DNA chip assay was higher than that for FOBT.

Sensitivity of the DNA chip assay and FOBT under several tumor conditions. It has been reported that patients with CRC with tumors less than 35 mm in diameter ('small' sub-group), located in the cecum, ascending colon or transverse colon (right colon subgroup), invading up to the *muscularis propria* (surface tumor subgroup), and metastasizing to neither lymph nodes nor other organs ('early' subgroup) frequently have false-negative results for FOBT (Table IV). The sensitivity of the DNA chip assay was higher than that of FOBT in all of these subgroups. In particular, the sensitivities of the DNA chip assay in both the surface tumor and 'early' subgroups were significantly higher than those of FOBT ($p=0.023$ and 0.019).

Discussion

FOBT fails to detect CRC in about half of all patients with tumors measuring less than 35 mm in diameter, located in the

Table II. *Genes highly expressed in fecal RNA from patients with colorectal cancer (CRC) compared to those in healthy controls.*

Gene symbol	*p*-Value	DNA sequence of chip probe
CEBPB	9.36E-12	CCGGTTTCGAAGTTGATGCAATCGGTTTAAACATGGCTGAACGCGTGTGTACACGGGACTGACGCAACCC
FCGR3A	3.42E-11	CAACCACAAGCACACAGGAAGGAAAGCGCAGGAGGTGAAAATGCTTTCTTGGCCAGGGTAGTAAGAATT
PFKFB3	4.44E-11	GAGCCTCCTATGTGTGACTTATGACTTCTCTGTGTTCTGTGTATTTGTCTGAATTAATGACCTGGGATAT
IL8	1.01E-10	ATTTTAATTGAACTAACAATCCTAGTTTGATACTCCCAGTCTTGTCATTGCCAGCTGTGTTGGTAGTGCT
SOD2	5.40E-10	GCCTTATTCCACTGCTGGGGATTGATGTGTGGGAGCACGCTTACTACCTTCAGTATAAAAATGTCAGGC
RGS2	7.88E-10	CTGGGATTATGTGGCCTTAGGTAGCTGGTTGTACATCTTTCCCTAAATCGATCCATGTTACCACATAGT
PPIF	1.21E-09	GTTGAACCTGGGAACAAACCTCACTTGAGCTGTGCCTAGACAATGTGAATTCCTGTGTTGCTAACAGAAG
BCL2A1	1.66E-09	GTTGTGTCCGTAGACACTGCCAGAACACTATTCAACCAAGTGATGGAAAAGGAGTTTGAAGACGGCATCA
SRGN	4.28E-09	AGGATCTGGGAGTGGCTTCCTAACGGAAATGGAACAGGATTACCAACTAGTAGACGAAAGTGATGCTTT
CSF3R	5.88E-09	GATCATGCTCCATCCAGCCCCACCCAATGGCCTTTTGTGCTTGTTTCCTATAACTTCAGTATTGTAAAC
NFKBIA	9.32E-09	TCGAGTGACTGACCCCAGTGGTATCCTGTGACATGTAACAGCCAGGAGTGTTAAGCGTTCAGTGATGTG
S100A9	9.08E-08	GGCCTGTTATGTCAAACTGTCTTGGCTGTGGGGCTAGGGGCTGGGGCCAAATAAAGTCTCTTCCTCCAA
S100A8	3.32E-07	AGAAAGCCTTGAACTCTATCATCGACGTCTACCACAAGTACTCCCTGATAAAGGGGAATTTCCATGCCG
ITM2B	5.72E-07	AAGGAAACTTACAAACTGCAACGCAGAGAAACTATTAAAGGTATTCAGAAACGTGAAGCCAGCAATTGT
IL1B	4.60E-06	ACCAAAGGCGGCCAGGATATAACTGACTTCACCATGCAATTTGTGTCTTCCTAAAGAGAGCTGTACCCA
S100A11	8.68E-06	ATCTCCACAGCCCACCCATCCCCTGAGCACACTAACCACCTCATGCAGGCCCCACCTGCCAATAGTAAT
ITM2B	1.15E-05	AAATGGACCACAGTGACTTATTTGTAGTTGTTAGTTGCCCTGCTACCTAGTTTGTTAGTGCATTTGAGCA
TALDO1	1.20E-05	CCGCCGGCCAGCTGGGATCTGACTGCACGTGGCTTCTGATGAATCTTGCGTTTTTTACAAATTGGAGCA
PLEK	3.04E-05	TCTGAGAGGGGCTGTTAGCATTGCGCAGCATCTTCAGTTCTCCAGTAAATGATATTGCGTTCGTGCCTC
FTL	8.84E-05	GAAGTGAAGCTCATCAAGAAGATGGGTGACCACCTGACGAACCTCCACAGGCTGGGAGGCCCAGAGGCTG
SAT1	1.22E-04	TACAGGGGCCTGGTCCGCAAAGGGAAGAAAAGCAAAAGACGAAAATGGCTAAATTCGTGATCCGCCCAGC
CCL3	1.67E-04	GATTGTTTGCTCTGAGAGTTCCCCTGTCCCCTCCCCCTTCCCTCACACCGCGTCTGGTGACAACCGAGTG
BCL3	3.92E-04	CGTAACGGGCACGGATCACGATGTAAATTATTAAGCATTTTGGTTGGATTTCTTTTGTAATAAACTATTT
ENO1	6.44E-04	CCGGGGTGGCCACAGGCTAGATCCCCGGTGGTTTTGTGCTCAAAATAAAAAGCCTCAGTGACCCATGAG
ZFP36L1	8.20E-04	TAAGTGGGGAGGAAGGGGAAGCTAGATGGACTAGGAGAGACTTGATTTTGGTGCTAAAGTTCCCCAGTTC
FTL	8.64E-04	GAAGTGAAGCTTATCAAGAAGATGGGTGACCACCTGACCAACCTCCACAGGCTGGGTGGCCCGGAGGCTG
RPS26	1.18E-03	TGCTGCCCCACGTCCCCCACCAAAGCCCATGTGAGGAGCTGAGTTCTTAAAGACTGAAGACAGGCTGTTC
H3F3A	1.22E-03	TTTATCATACAGTAGATTCCATCCATTCACTATACTTTTCTAACTGAGTTGTCCTACATGCAAGTACATG
EMILIN1	1.44E-03	GCTCCTGTCCGTCCCCTCTCGGTTCCTTCCGCTCTCCTCTTCACTTCTGAGGGGGTTTGTTCTCTGTGCC
PYY	1.53E-03	TGCGGCAGTGCAGCCCTTGGGACTTCCCTCGCCTTCCACCTCCTGCTCGTCTGCTTCACAAGCTATCGCT
CMTM6	1.93E-03	GTTGTCTTTAGGTGGTCCTGAATCTTGGGCCCTTTTGATTGTGAATACTGTGTAGCAGGATCTTGAGAG
SPRR1A	4.48E-03	ATTCTCAGCAGCAGAAGCAGCCTTGCACCCCACCCCCTCAGCCTCAGCAGCAGCAGGTGAAACAACCTTG
ACTB	5.60E-03	GCCCAGTCCTCTCCCAAGTCCACACAGGGGAGGTGATAGCATTGCTTTCGTGTAAATTATGTAATGCAAA
CLTB	8.44E-03	CCCTCCTGGCCCTCTGGTCCAGCCCCTCACGCCTCCTCTCAGTCTACTCAATTGTGACTGTCCCTCCTGA
AQP8	8.56E-03	GTTTCTGCACATCAGCTCATTTCCCGCACCCCATTTCTTGCTTGATTGCTTTGTTGGGGGCCTGGCCACT
KRT8	1.48E-02	CTAGCCCTCAGCCCACCTGGGGAGTTTACTACCTGGGGACCCCCCTTGCCCATGCCTCCAGCTACAAAAC
MAPK8IP2	1.95E-02	CTTTTCCTCAGATCCGTTCTTTCTCTGTGTTGTCCTCCTCCTTCCCTTCCCAGTCTCCCTTTTCTCTCTC
ARPC3	2.08E-02	GAAGAGCATGTCTTTACTTGAAAAACTCTTGATCAAGAATTTGGGTGGGAGAAAAGAAAGTGGGTTATCA
ZG16	2.25E-02	TCTGCACCCTGGGGAATCAGTGATCCAGGTTTCTGGGAAGTACAAGTGGTACCTGAAGAAGCTGGTATTT
B2M	2.99E-02	TGGACATGATCTTCTTTATAATTCTACTTTGAGTGCTGTCTCCATGTTTGATGTATCTGAGCAGGTTGCT
HSPA1A	3.74E-02	CCCACCATTGAGGAGGTAGATTAGGGGCCTTTCCAAGATTGCTGTTTTTGTTTTGGAGCTTCAAGACTT
CAMK1	4.03E-02	GAGTTTCTCTGCCCTGTCCCCTCCTCACCTGCTTCCCTACCACTCCTCACTGCATTTTCCATACAAATG
GBP2	4.64E-02	GCAAAATTTGCCTGTCCAGCTCCCTCTCCCCAAGAAACAACATGAATGAGCAACTTCAGAGTGTCAAAC

Differences in gene expression between the patients and healthy controls of the training set were analyzed by the Wilcoxon signed-rank test (unpaired, two-sided). The *p*-values were adjusted for multiple testing by Bonferroni's correction and values less than 0.05 were considered to denote statistical significance.

right side of the colon, invading up to *muscularis propria*, and metastasizing to neither lymph nodes nor other organs. Colonoscopy is recognized worldwide as the golden standard for the diagnosis of CRC. However, it is difficult to reach the ascending colon or cecum with the scope in approximately 10% of the examinees due to anatomical or technical reasons (30). Among patients undergoing resection for right-sided CRC, the reported miss rate of colonoscopy for the diagnosis of CRC in usual clinical practice is 4.0% (31). In fact, patients with right-sided CRC are frequently diagnosed only at an advanced stage (32, 33). In this context, we investigated in this study if use of the DNA chip assay can overcome some of these problems. Although no significant difference in the sensitivity was found between the DNA chip assay and

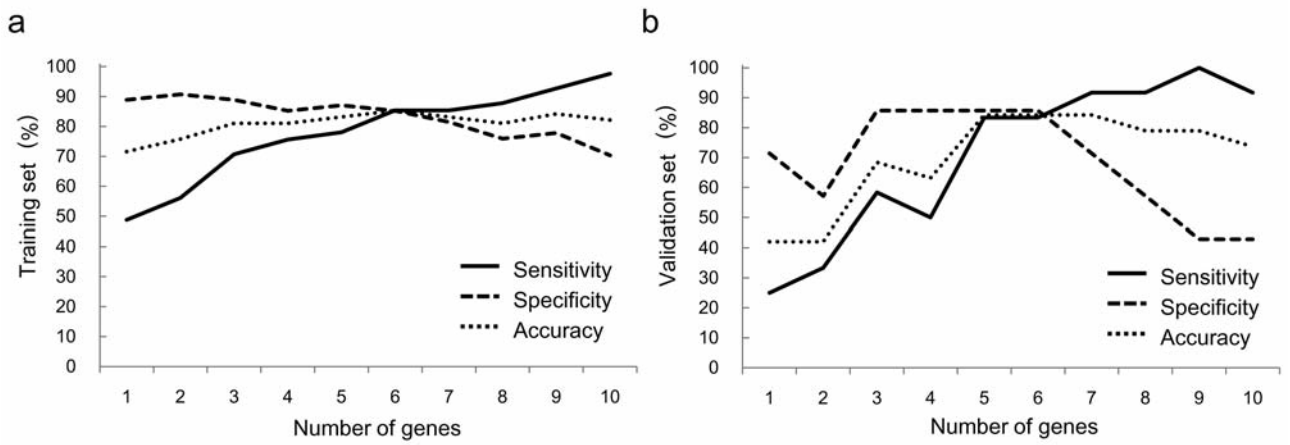

Figure 1. *Sensitivity, specificity, and accuracy of the DNA chip assay in the training (a) and validation (b) sets as calculated by support vector machine analysis using expression of different numbers of genes.*

Table III. *Sensitivity, specificity and accuracy of the DNA chip assay using six genes and fecal occult blood test (FOBT).*

	Training set			Validation set		
Assay name	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy
DNA chip assay	85.4% (35/41)	85.2% (46/54)	85.3% (81/95)	83.3% (10/12)	85.7% (6/7)	84.2% (16/19)
FOBT	53.7% (22/41)	98.1% (53/54)	78.9% (75/95)	66.7% (8/12)	100% (7/7)	78.9% (15/19)

DNA chip assay: *CEBPB* (CCAAT/enhancer binding protein, beta); *FCGR3A* (Fc fragment of IgG, low affinity IIIa, receptor); *PFKFB3* (6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3); *IL8* (interleukin 8); *SOD2* (superoxide dismutase 2); and *RGS2* (regulator of G-protein signaling 2) were used; Sensitivity: rate of patients with CRC with positive test (true-positive patients) out of all patients with CRC; Specificity: rate of healthy controls with negative test (true-negative controls) out of all healthy controls; Accuracy: rate of true-positive patients and true-negative controls in all participants.

Table IV. *Sensitivity of the DNA chip assay and fecal occult blood test (FOBT) for several tumor subgroups.*

	Sensitivity (number of patients with positive test/all patitents)						
	Training set		Validation set		Both sets		
Tumor subgroup	DNA chip assay	FOBT	DNA chip assay	FOBT	DNA chip assay	FOBT	*p*-Value
Size: Small	73.7% (14/19)	42.1% (8/19)	88.9% (8/9)	66.7% (6/9)	78.6% (22/28)	50.0% (14/28)	0.051
Location: Right colon	100% (6/6)	50.0% (3/6)	80% (4/5)	40.0% (2/5)	90.9% (10/11)	45.5% (5/11)	0.067
Depth: Surface	81.3% (13/16)	43.8% (7/16)	100% (6/6)	60.7% (4/6)	86.4% (19/22)	50.0% (11/22)	0.023
Stage: Early	84.0% (21/25)	44.0% (11/25)	75.0% (6/8)	75.0% (6/8)	81.8% (27/33)	51.5% (17/33)	0.019

DNA chip assay: six genes (*CEBPB*; *FCGR3A*; *PFKFB3*; *IL8*; *SOD2*; and *RGS2*) were used; Small: diameter of the tumor was less than 35 mm; Right colon: tumor was located in cecum; ascending colon or transverse colon; Surface: tumor was invading up to the muscularis propria (T1 and T2); Early: tumor not metastasizing to lymph nodes or other organs (Dukes' stage A and B); Both sets: combined training set and validation set; *p*-value: analyzed by χ^2 test; with $p<0.05$ considered to denote statistical significance.

FOBT for the right colon subgroup because of the small size of the population, right-sided CRC was detected by the DNA chip assay in 90% of the patients. This result is important in that the detection rate of patients with right-sided CRC could be increased and the mortality of CRC could be reduced if the chip assay were used for CRC screening. Therefore, a large-scale randomized controlled trial is necessary to determine this.

In general, the recovery rate of RNA originating from CRC cells in fecal samples is less than 1% and almost all of the fecal RNA is from bacteria (22). Therefore, cancer-derived RNA is difficult to detect by gene expression analysis using conventional DNA chip. In this study, we used a highly sensitive DNA chip, the 3D-Gene® (25). This DNA chip can detect the RNA of even those genes that are expressed in very small amounts. Furthermore, the fecal samples are dipped in RNAlater® Solution to protect free RNAs from the nucleases contained in feces (34, 35). Regarding the optimal fecal storage condition for obtaining intact RNA, 0.5 g fecal samples should be dipped in 10 ml of RNAlater® Solution and stored at 4˚C for no longer than three days according to our preliminary simulation study (data not shown).

Based on the results of the DNA chip assay, we identified six genes as candidates for the detection of CRC. The protein encoded by the *CEBPB* gene is a member of the basic leucine zipper (bZIP) transcription factor and plays a role in inflammation, cell survival, apoptosis, and tumor transformation (36). The expression of *CRBPB* was positively correlated with that of keratin 23 (KRT23) and increased in CRC tumor compared to that in normal mucosa (37). The *FCGR3A* gene encodes the receptor for the Fc portion of immunoglobulin G and is involved in the removal of antigen–antibody complexes from the circulation, as well as other antibody-dependent responses. The protein encoded by this gene is expressed on natural killer cells and macrophages. The *FCGR3A*-V158F polymorphism has been shown to influence the efficacy of cetuximab in patients with epidermal growth factor receptor-positive CRC *via* antibody-dependent cell-mediated cytotoxicity (38). Induction of *PFKFB3* mRNA expression by hypoxia has been reported to be strong in several types of tumor, such as colon, breast, ovarian and thyroid tumors (39). IL8 is one of the chemokines most significantly up-regulated in CRC (40). IL8 has an important role in initiating inflammation, therefore, it may contribute to colorectal carcinogenesis *via* inducing chronic colonic inflammation (41). SOD2 is a key anti-oxidant enzyme that is known to be an effective scavenger of superoxide radicals. CRC cells appear to have high levels of SOD2 expression in order to adapt to the high levels of oxidative stress they experience (42). RGS2 is an inhibitor of G-protein-coupled receptor and is expressed in several tumor types (43). In this study, CEBPB, CRBPB, IL8, SOD2 and RGS2 may have originated from CRC cells, while FCGR3A could have derived from intestinal bleeding and not the CRC cells.

The specificity of our DNA chip assay was unfortunately not adequate. However, it should be noted that the use of this method effectively allowed for detection of patients with small, right-sided, surface tumor, and early-stage CRC. Although the sample size of this study was small, based on results of this study, further evaluation of the DNA chip assay for CRC screening appears to be warranted.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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