The Relationship between the Methylated Septin-9 DNA Blood Test and Stool Occult Blood Test for Diagnosing Colorectal Cancer in Taiwanese People

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Background: Colorectal cancer (CRC) is a common and lethal disease in the world. There is an increasing number of cases in Taiwan and a higher rate at advanced stages. The immune fecal occult blood test (iFOBT) has been used as a screening method in Taiwan for years. A new novel diagnostic tool, the Methylated Septin-9 (MS-9) DNA blood test, had been reported to have high sensitivity and specificity for CRC detection. There are no available data in Taiwan, so we conducted this prospective randomized trial to investigate the relationship among the MS-9 DNA blood test, iFOBT, and a combination of the two tests for diagnosing CRC in Taiwanese people. **Methods:** From July 1, 2012 to December 31, 2013, we prospectively selected 60 plasma samples from patients who were diagnosed with CRC and otherwise, the healthy group by colonoscopy in our hospital. Patients were divided into the CRC group and healthy group. CRC stages 0, I, II and stages III and IV were separately

analyzed. We calculated the sensitivity and specificity of each group to determine the relationship among the MS-9 DNA blood test, iFOBT, and a combination of the two tests for diagnosing CRC in Taiwanese people. **Results:** The results of the MS-9 DNA blood test for the 60 samples were divided into three groups, and the sensitivity as well as the specificity of the MS-9 DNA blood test to detect CRC were 47% and 89%, respectively. The results of iFOBT were also divided into three groups, and had higher sensitivity (84%) but lower specificity (55%) using iFOBT to detect CRC. Higher rates could be predicted to detect CRC if both the tests were positive. **Conclusions:** A combined MS-9 DNA blood test and iFOBT may help in a higher detection rate of CRC. It could be offered to individuals who are unwilling or unable to undergo colonoscopy. Further large prospective, randomized studies are needed in the future. *J. Clin. Lab. Anal.* 31:e22013, 2017. © 2016 Wiley Periodicals, Inc.

Key words: colorectal cancer; immune fecal occult blood test; methylated Septin-9 DNA blood test

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy in the world (1). There are an increasing number of cases in Taiwan and a higher rate at an advanced stage. As the early symptoms of CRC are obscure, it is easily overlooked by the patients and physicians, which delays the timing of early diagnosis and treatment. However, if early detection of CRC

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can be completed, it has a higher chance of being cured. As the disease is common and lethal, we are hoping to rely on early detection to improve the patient's survival rate. The screening tests, in particular, are the keys to decrease CRC-related mortalities by detecting tumors at an earlier stage. The commonly used screening methods, such as the fecal occult blood test, CT colonography, flexible sigmoidoscopy, and colonoscopy all have limitations (2–5). The Ministry of Health and Welfare in Taiwan has promoted free CRC screening since 2004, using the immune fecal occult blood test (iFOBT), for no family or personal history of people above the age of 50–69. According to the statistics, fecal occult blood test screening every 1–2 years could have reduced the incidence of CRC by about 18–33%. Some studies suggest that uptake, adherence, and follow-up rates for fecal-based tests are disappointing (6, 7). From a clinical point of view, the accuracy of iFOBT, although not as invasive as sigmoid colonoscopy or colonoscopy, is a simple and rapid method of CRC screening. The public preference for a non-invasive screening method might be higher than for other methods. If we can collect a simple blood test to do the CRC screening, it will encourage more individuals for examination, and could significantly reduced CRC mortality. The results of increasing screening rates will also be reflected in the medical cost savings (8, 9). As more CRC will be diagnosed in the early stages and costly chemotherapy can also be avoided (10). The MS-9 DNA blood test has been reported to have high sensitivity and specificity for CRC detection (11–15). However, there is no available data in Taiwan. Therefore, this prospective randomized trial will be to determine the relationship among the MS-9 DNA blood test, iFOBT, and a combination of the two tests for diagnosing CRC in Taiwanese people.

METHODS

Patients

A total of 2,456 patients who underwent colonoscopy in our hospital from July 1, 2012 to December 31, 2013 were included in the study. The reasons for colonoscopy included healthy examination, iFOBT positive, previous history of colonic polyp, and CRC, family history of CRC, recent signs of GI bleeding, bowel habit change, and unknown reason of abdominal pain. We prospectively, randomly selected 60 MS-9 DNA plasma samples and stool occult blood tests from patients who had CRC during colonoscopy (CRC group, proved by pathologic report) and a healthy group (no CRC during the colonoscopy).

Patients with previous CRC (n : 85), other gastrointestinal tract malignancy (n : 29), incomplete colonoscopy study (n : 123), or refused the MS-9 DNA blood test (n : 434), iFOBT (n : 377), adenoma, hyperplastic polyps, and negative finding during the colonoscopy (n : 1,348) were excluded from the analysis (Fig. 1). Finally, 60 patients were enrolled and divided into a healthy group (n : 9) and CRC group (n : 51). CRC stages 0, I, and II, (stage 0 – carcinoma in situ) and CRC stages III and IV were separately analyzed. We used a commercially available assay, three independent blinded laboratories assayed plasma DNA of all samples. Then we analyzed the sensitivity and specificity of each group to determine the relationship among the MS-9 DNA blood test, iFOBT, and a combination of the two tests for diagnosing CRC in Taiwanese people.

Human Plasma Methylated Septin 9 DNA Blood Test

In Abbott MS-9 test reagents kits and the Abbott real-time PCR platform for the qualitative detection were used to see if the specimen Septin 9 gene methylation situation had occurred which differs from the Epi proColon test used in several previous studies. The Epi proColon test is designed as a duplex real-time PCR for the methylated Septin 9, comprising the Epi proColon Plasma Quick kit, the Sensitive PCR kit, and Control kit. Briefly, free circulating DNA from 3.5 ml of plasma, obtained through pooling of two aliquots of subject plasma each containing ~2 ml, was captured on magnetic beads, purified, and concentrated. For the Abbott MS-9 DNA blood test method, it was performed following the manufacturer's instruction at a qualified laboratory which cooperates with our hospital. The Abbott m24sp automated platform is used to process plasma samples or manually operate nucleic acid extraction and then the Abbott m2000rt is used for amplification and detection. DNA must first be extracted from the plasma and then the Abbot bisulfite modification kit is used to modify DNA and purify DNA from the modification reaction. DNA extraction and purification utilize the Abbott mSample Preparation System DNA reagent kits. When the 96-well optical reaction plate preparation of a PCR reaction is complete, the reaction plate needs to be sealed and moved into an Abbot m2000rt to process the amplification and immediately detect the fluorescence volume. M2000rt computer software can automatically output patient outcomes. The Abbott colorectal MS-9 immediate inspection test is a parallel test which can simultaneously monitor the presence of Septin 9 methylation and β -actin. B-actin is the internal quality control of the sample which can

Fig. 1. Showed the flow diagram of the study method.

assess sample extraction, bisulfite modification, and the polymerase chain lock amplification reaction. Sodium bisulfite is used for analyzing the individual cytosine methylation status residues on the DNA sequence. Sodium hydrogen sulfite can convert cytosine into uracil, leaving 5-methylcytosine residues without change. A polymerase chain reaction was designed to amplify the methylated target sequences of Septin 9 and use specific methylated probes for detection. β -actin amplification products are generated from β -actin primer set sequences and β -actin-specific probe detection is used. Methylated Septin 9 and the β -actin probe are labeled in different fluorescence and can independently collect their signals.

In summary, the MS-9 DNA blood test is qualitative only. We collected 15-ml peripheral blood samples in ethylenediamine tetraacetic acid (EDTA) tubes before colonoscopy. The amount of blood is thought to be a major determinant in the performance of the MS-9 DNA blood test. However, one recent study claimed age, diabetes, and arthritis could also affect the results (16). We did not analyze the MS-9 DNA blood test if the blood amount was less than 15 ml. The Abbott real-time PCR platform for the qualitative detection was used to see if the specimen Septin 9 gene methylation situation had occurred. The computer software automatically determines data results to be positive or negative. The test is performed as a

triplicate PCR for each specimen. As long as the specimen is shown in one of the three internal quality control clearances and a positive MS-9 signals, it can be determined as positive; even if the other replicate is not positive result. If the specimen repeats the three tests and the three internal quality controls give clearance and negative MS-9 results, it can be judged to be negative.

Immune Fecal Occult Blood Test (iFOBT)

Immune fecal occult blood test was provided with a fecal sample for immune occult blood testing before colonoscopy. Diet and medication were not restricted before providing the fecal sample. The qualitative iFOBT kits were provided by Taipower, Inc. (Taichung, Taiwan). The tests were performed in the Clinical Laboratory of Show Chwan Memorial Hospital, according to the manufacturers' instructions. The positive threshold of iFOBT was 0.2 µg/ml.

Statistical Analysis

The sensitivity and specificity for each test were calculated with 95% confidence intervals (CI) based on the distribution. Chi-squared test was used to compare the detection rates of the MS-9 DNA blood test and iFOBT for CRC between ages, genders, and location. Comparison of the CRC detection rates was based on an ROC curve according to the sensitivity and specificity of these two tests. A statistically significant difference was established when $P < 0.05$. AUC (area under curve) > 0.7 indicated acceptable discrimination, whereas > 0.8 indicated excellent discrimination.

RESULTS

Table 1 shows the clinical characteristics of 60 enrolled patients, including age, gender, cancer location, and stage between the results of the MS-9 DNA blood test. Table 2 shows the clinical characteristics between the results of iFOBT, whereas Table 3 shows the clinical characteristics between the results of the combination of the two tests. The results of the MS-9 DNA blood test, iFOBT, and the combination of the two tests showed no significant statistical difference, including age, gender, and cancer location, except cancer stage.

The positive MS-9 DNA blood tests for the 60 samples numbered 25, divided into three groups. One in the healthy group, eight in CRC stages 0, I, and II, and sixteen in CRC stages III and IV (Table 1).

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the MS-9 DNA blood test to detect CRC were 47%, 89%,

TABLE 1. Clinical characteristics between the results of the MS-9 DNA blood test

	MS-9(+) (<i>n</i> = 25)	MS-9(−) (<i>n</i> = 35)	<i>P</i>
Age (Mean ± SD)	64.20 ± 12.4	66.9 ± 12.3	0.411
Gender			
Male	15 (60.0%)	20 (57.1%)	0.825
Female	10 (10.0%)	15 (42.9%)	
Location			
Left-side colon	19 (76.0%)	28 (80.0%)	0.711
Right-side colon	6 (24.0%)	7 (20.0%)	
Cancer stage			
Healthy group	1 (4.0%)	8 (22.9%)	0.004
Stages 0, I, II	8 (32%)	15 (42.8%)	
Stages III, IV	16 (64%)	12 (34.3%)	

TABLE 2. Clinical characteristics between the results of the iFOBT

	iFOBT(+) (<i>n</i> = 47)	iFOBT(−) (<i>n</i> = 13)	<i>P</i>
Age (Mean ± SD)	65.4 ± 12.0	67.2 ± 13.9	0.633
Gender			
Male	30 (63.8%)	5 (38.5%)	0.101
Female	17 (36.2%)	8 (61.5%)	
Location			
Left-side colon	37 (78.7%)	10 (76.9%)	1.000
Right-side colon	10 (21.3%)	3 (23.1%)	
Cancer stage			
Healthy group	4 (8.5%)	5 (38.5%)	0.002
Stages 0, I, II	16 (34.0%)	7 (53.8%)	
Stages III, IV	27 (57.5%)	1 (7.7%)	

96%, and 22%. Meanwhile, the results of iFOBT for the 60 samples were also divided into three groups according to the CRC stage, with 4 positive in 9 samples for the healthy group, 16 positive in 23 samples for CRC stages 0, I, and II, and 27 positive in 28 samples for CRC stages III and IV (Table 2). The sensitivity, specificity, PPV, and NPV of iFOBT were 84%, 55%, 91%, and 38% (as shown in Table 4).

The combination of the two tests showed that the positive and negative findings in diagnosing CRC were 23 and 11 samples, respectively. The MS-9 DNA blood test positive and iFOBT negative included two samples, whereas the MS-9 DNA blood test negative and iFOBT positive included 24 samples (Table 3).

An ROC curve (AUC = 0.766) was used to predict CRC based on the sensitivity and specificity of the MS-9 DNA blood test and iFOBT. Higher rates could be predicted to detect CRC if both tests were positive (as shown in Fig. 2).

DISCUSSIONS

Many studies have confirmed if the screening test is done, the early stages of CRC can be effectively

TABLE 3. Clinical characteristics between the results of the MS-9 DNA blood test and iFOBT

	MS-9(+)/iFOBT(+) (n = 23)	MS-9(-)/iFOBT(+) (n = 24)	MS-9(+)/iFOBT(-) (n = 2)	MS-9(-)/iFOBT(-) (n = 11)	P
Age (Mean $\pm$ SD)	63.8 $\pm$ 12.9	66.9 $\pm$ 11.2	69.0 $\pm$ 4.2	66.9 $\pm$ 15.2	0.805
Gender					0.280
Male	15 (65.2%)	15 (65.2%)	0 (0.0%)	5 (45.5%)	
Female	8 (34.8%)	9 (37.5%)	2 (100.0%)	6 (54.5%)	
Location					0.756
Left-side colon	18 (78.3%)	19 (79.2%)	1 (50.0%)	9 (81.8%)	
Right-side colon	5 (21.7%)	5 (20.8%)	1 (50.0%)	2 (18.2%)	
Cancer stage					0.000
Healthy group	1 (4.3%)	3 (12.5%)	0 (0.0%)	5 (45.5%)	
Stages 0, I, II	7 (30.4%)	9 (37.5%)	1 (50.0%)	6 (54.5%)	
Stages III, IV	15 (65.2%)	12 (50%)	1 (50.0%)	0 (0.0%)	

TABLE 4. Sensitivity, specificity, PPV, and NPV of the MS-9 DNA blood test and iFOBT

	MS-9 (%)	iFOBT (%)
Sensitivity	47	84
Specificity	89	55
PPV	96	91
NPV	22	38

Fig. 2. Showed an ROC curve, area under curve (AUC) = 0.766.

treated, and mortality can be reduced (17–21). Of all the screening tests, colonoscopy is proven to have the highest sensitivity and specificity. However, on the basis of some reports, only half of potential patients aged over 50 complete screening (22). The major obstacle to screening by colonoscopy is patient

compliance with the screening recommendations (23). Although most doctors may recommend patients undergo screening, the patients still makes the final decision for screening based on their individual preferences (24). Time-consuming and invasive colonoscopy in checking for potential complications is the main reason for not accepting colonoscopy referral procedures. In recent years, we found the rate of stool testing for CRC was still low in Taiwan. According to the statistical data in 2011 from the Ministry of Health and Welfare of Taiwan, the screening rate was lower than 33% and the CRC screening detection rate was about 5%, which was still low. There are two main variables in analyzing the reasons. In addition to the detection of multiple stool samples and specimen processing requirements, patient compliance is also a challenge. To increase the screening rates and find more early stage of CRC, the authorities concerned adjusted the screening age from 50–69 in 2004 to 50–74 in 2013.

The Septin 9 gene encodes a mammalian septin protein involved in many cellular processes. In the cell division cycle mutant yeast, Septins were originally detected. In neoplasia, neurological, and infectious diseases, Septin is known to be implicated. When Septin 9 gene is methylated, disruption of the action of Septin 9 results in incomplete cell division, which will contribute to the tumor growth (25). Septin 9 has been shown to be related to the proto-oncogene, suggesting a role in tumorigenesis (26, 27). In addition to CRC, the Septin 9 gene has also been shown to be closely involved in breast and ovarian cancers.

This study shows that the positive MS-9 DNA blood test or iFOBT tests finding has more chance of being located on the left side of the colon than the right side. Some authors claimed anatomical factors such as a lower colonic lumen diameter may lead to the increasing number of diagnosed left-sided CRC. It is generally known that right-sided CRC has a worse prognosis than left-sided CRC (28). Whereas

right-sided CRC display elevated gene expression levels of cell cycle control and signaling genes, left-sided CRC shows reduced expression of tumor suppressor genes and cytokeratin 20 and elevated expression of COX-1 and genes that promote stromal expansion (29, 30). The differences in methylation caused by epigenetic factors result in tumorigenesis between left- and right-sided CRC. Some studies reveal left-sided CRC can display a mutated phenotype, whereas right-sided CRC displays as a hypermethylated phenotype (31–33). This study's sample size is not presently large enough due to the high cost of the MS-9 DNA blood test. It is considered that the MS-9 DNA blood test can be performed as well as iFOBT for CRC detection, but it lacks the potential therapeutic advantages and lower sensitivity of early CRC. In this study, the sensitivity of iFOBT was much higher than the MS-9 DNA blood test for early CRC detection probably due to the MS-9 DNA blood test of early CRC remaining negative. This means that the Septin 9 gene of early CRC is not methylated. Other colonic neoplasm, like adenoma and hyperplastic polyps were not included in this study. Septin 9 is methylated in adenomas, as shown by tissue studies. However, blood-based detection is low, perhaps due to lack of access of adenomas to the blood supply.

We could not collect as many samples as the previous study did in PRESEPT study (34) due to a limited budget. However, the sensitivity and specificity of 47% and 89% were similar to the PRESEPT study. This was thought to be a low CRC detection rate in our study, especially for early-stage CRC. For CRC stages 0 and I, the MS-9 DNA blood test detection rate was only 7.7%. Despite this, in our study combining the MS-9 DNA blood and iFOBT test, it showed both positive results had higher CRC detection rates than both negative results. We believe the blood-based CRC check, which we can collect by blood specimens in primary care every 2 or 3 years, will attract those who do not implement the provisions of CRC screening guidelines for screening of subjects. If the iFOBT and MS-9 DNA blood test both show positive results, colonoscopy or even a CT or PET scan will be strongly advised for further study. We look forward to more in-depth study on this blood-based test with the help, it will encourage the general average risk subjects to be screened and get a better understanding. It could be offered to individuals who are unwilling or unable to undergo colonoscopy. When the screening is out of patients' willingness, MS-9 DNA blood test can be an alternative choice for screening tools for CRC, even with the low detection rate of adenoma and early CRC. It is known that CEA is the most widely used serum tumor marker for CRC monitoring. If the MS-9

DNA blood test could be quantitatively analyzed, the further relationship with the CEA level, before and after surgery, might also be analyzed.

At present, people are posed an increased health threat with CRC. A less intrusive and more direct way to screening is aimed for in this study because a separate drawing of blood is more convenient, and thus it can be expected to have an increased number of CRC screening rates and to achieve early detection of CRC, to achieve better health.

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