Format for ANSWERING REVIEWERS

February 12, 2014

Dear Editor,

Title: Detection of early colorectal cancer via promoter hypermethylation of Wnt antagonist genes in fecal samples

Author: Hu Zhang, You-Qing Zhu, Ya-Qiong Wu, Ping Zhang, Jian Qi

Name of Journal: World Journal of Gastroenterology

ESPS Manuscript NO: 7200

The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer
(1) In the Materials and Methods section, the authors should describe in more details their patients, (actually they are presented only in the Results section of the paper) in order to let the reader know what they are talking about. A table may be useful there.
Answer: seen in Table 1 in the paper.

(2) In the results section you mention a group of controls but they are not mentioned in the methods section. Which were the criteria you used for defining normal controls.
Answer: The normal subjects were the ones with the normal colorectal mucosa examined by endoscopy or undergoing surgery.

(3) Results should be reported for patients, not for lesions.
Answer: Yes, We think your opinion is correct.

(4) The name Dukes should be used for staging classification (not Duck or others).
Answer: Yes, We think your opinion is correct.

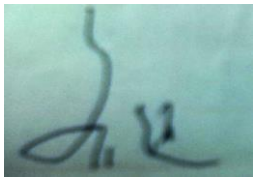
(5) FOBT was performed with or without diet with meat restriction?
Answer: There are two types of FOBT available for CRC screening: the guaiac and the immunochemical tests. The first test needs dietary restrictions and has low sensitivity, while the immunochemical test is specific for human haemoglobin and does not require any dietary restrictions. In our study, we use the immunochemical test for screening, therefore, FOBT was performed without diet restriction.

(6) I think that the statistical analysis is incomplete. The Sensitivity, Specificity, PPV and NPV as well as the accuracy of the evaluated tests should be calculated. It will not be hard to do since in all cases a gold-standard is available.
Answer: Yes, We think your opinion is correct. We add the statistical in table 1 in my paper.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



Jian Qi, MD, PhD
Zhongnan Hospital of Wuhan University
Wuhan
Hubei Province
China
Fax: +86-27-87336141
E-mail: qiqidelizi @ aliyun.com

Name of journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 7200

Columns: BRIEF ARTICLE

Detection of early colorectal cancer via promoter hypermethylation of Wnt antagonist genes in fecal samples

Running title: ***Zhang H, et al., Hypermethylated gene in stools from CRC***

Hu Zhang, You-Qing Zhu, Ya-Qiong Wu, Ping Zhang, Jian Qi

Hu Zhang, You-Qing Zhu, Jian Qi, Department of Gastroenterology and Clinical Center of Intestinal and Colorectal Diseases of Hubei Province, Zhongnan Hospital of Wuhan University, Wuhan, 430071, Hubei Province, China; **Hu Zhang, Ping Zhang,** Department of Gastroenterology, Hubei Provincial Corps Hospital, Chinese People's Armed Police Force, Wuhan 430061, China.

Author contributions: Zhu YQ, Qi J and Zhang P designed this study and critically revised the article; Zhang H and Zhang P were responsible for data analysis and interpretation of the results; Zhang H drafted the manuscript; all authors read and approved the final manuscript to be published.

Supported by The National Natural Science Foundation of China Youth Science Fund, No. 81101868 and The Natural Science Foundation of Hubei Province of China, No. 2011CDB505

Correspondence to: Ping Zhang, PhD, Department of Gastroenterology, Hubei Provincial Corps Hospital, Chinese People's Armed Police Force, No 475 Minzhu Road, Wuchang District, Wuhan, 430061, China. Jian Qi, PhD, MD, Department of Gastroenterology, Zhongnan Hospital of Wuhan University, No 169 Donghu Road, Wuchang District, Wuhan, 430071, Hubei Province, China. qiqidelizl@aliyun.com.

Telephone: +86-27-87336141 **Fax:** +86-27-87336141

PURPOSE: To investigate the feasibility of detecting aberrantly hypermethylated Wnt-antagonist gene promoters (SFRP2 and WIF-1) in fecal DNA as non-invasive biomarkers for early colorectal cancer (CRC).

METHODS: The methylation-specific PCR assay (MSP) was performed to blindly analyze the methylation status of SFRP2 and WIF-1 gene promoters in fecal samples from 48 subjects with colorectal carcinomas, 35 with adenomas, 32 with hyperplastic polyps and 30

endoscopically normal subjects. Additionally, we compared the diagnostic efficiency of measuring the hypermethylated SFRP2 and WIF-1 genes in the feces to the fecal occult blood test (FOBT) for the early detection of CRC.

RESULTS: Hypermethylated SFRP2 was detected in the feces of 56.3% (27/48) of CRC cases, 51.4% (18/35) of adenoma cases and 12.5% (4/32) of patients with hyperplastic polyps. The hypermethylation of WIF-1 was detected in 60.4% (29/48), 45.7% (16/35) and 18.7% (6/32) of fecal samples from CRC, adenoma and hyperplastic polyp patients, respectively. At least one hypermethylated gene was detected in 81.3% (39/48) of CRC and 65.7% (23/35) of adenoma samples. In contrast, only one case of a hypermethylated WIF-1 gene was detected in normal fecal samples. Moreover, no significant associations were observed between SFPR2 and WIF-1 hypermethylation and clinicopathological features. Additionally, 81.8% of CRCs diagnosed as Dukes A stage or advanced adenomas had at least one hypermethylated gene detected, while the detection rate with the FOBT was only 31.8%, with $P < 0.001$.

CONCLUSIONS: Hypermethylation of the SFRP2 and WIF-1 genes in fecal DNA are novel and promising molecular biomarkers that have great diagnostic potential for early CRC.

KEY WORDS: Colorectal carcinoma; Secreted frizzled-related protein 2 (SFRP2); Wnt inhibitory factor-1 (WIF-1); Stool; Methylation

***Core tip*:** Current available approaches for the early detection of colorectal carcinoma (CRC) are suboptimal. The analysis of methylation markers in the stool as a non-invasive test is important for the early diagnosis of CRC. The epigenetic silencing of Wnt antagonist genes occurs in the early stages of CRC, and up to ninety percent of colorectal cancers result in the aberrant activation of Wnt signaling. In our study, we found that hypermethylation of the SFRP2 and WIF-1 genes in fecal DNA are novel and promising molecular biomarkers that have a great potential for the diagnosis of early CRC.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer-related death in Western countries [1], and the incidence rate has rapidly increased in Eastern countries in the past decade, especially in China [2]. The survival is closely related to CRC stage at the time of diagnosis.

A 5-year survival of stage I CRC has reached 90% [3], but less than 10% for CRC cases have distant metastases [4]. However, most CRC patients are diagnosed in the middle or late stages because no typical symptoms for the early stage of CRC exist [5]. Therefore, the diagnosis of CRC in early stage has great importance for reducing CRC mortality.

The critical target lesions have included the early stages of CRC and the highest risk of precancerous lesions [6]. Historically, colorectal adenoma has been recognized as the most important precancerous lesion of CRC. Early detection of CRC or the precancerous lesions is a major challenge to improve patient's survival and widen the window of therapeutic intervention.

Although the incidence of CRC in the United States has decreased by 2%-3% every year over the past 15 years, the discovery rate of early CRC is still less than 5% [6]. The current available approaches to early detection of colorectal tumor are suboptimal. With evolving insights into the molecular changes during CRC development, assays to detect tumor-derived DNA alterations in the feces are a promising approach that may improve screening effectiveness and user friendliness for the screening and early diagnosis of colorectal neoplasia.

Genetic alterations are believed to drive the histological progression of CRC in the adenoma-carcinoma sequence of colorectal cancer. The aberrant methylation of genes appears to act in concert with other genetic alterations and has been recognized as one of the most common molecular alterations in CRC. The epigenetic silencing of Wnt antagonist genes occurs in the early stages of CRC, and up to ninety percent of colorectal cancers result in the aberrant activation of Wnt signaling [7, 8]. The Wnt antagonists, secreted frizzled related protein gene family (SFRPs) and Wnt inhibitory factor-1 (WIF-1), have been found to be hypermethylated and are frequently not expressed in CRC cell lines and primary CRC samples [7, 9]. Our previous study demonstrated that Wnt-antagonists with promoter hypermethylation were frequently found in early CRC [9].

In this study, we extended the previous work and analyzed the methylation status of SFRP2 and WIF-1 genes in stools taken from CRC patients, from patients with benign colorectal diseases and from normal controls and then assessed the potential of fecal SFRP2 and WIF-1 gene hypermethylation as a non-invasive screening tool for CRC. Additionally, we compared the early CRC diagnostic efficiencies of the detection of hypermethylated SFRP2 and WIF-1 genes in the stool to the fecal occult blood test (FOBT).

MATERIALS AND METHODS

Stool sample collection

Stool samples were collected from 48 patients with sporadic CRC, 67 patients with benign colorectal diseases (35 colorectal adenoma and 32 hyperplastic polyps) and 30 endoscopically normal patients undergoing surgery and endoscopy at the clinic of the Zhongnan Hospital of Whan University between February 2012 and March 2013. The patients' clinicopathological features are listed in Table 1. All samples were stored at -80℃ until processing and all patient diagnoses were confirmed by pathology after their surgery or colonoscopy. None of the patients had received chemotherapy or radiation therapy prior to surgery. All matched samples were also analyzed with a FOBT (Fecal Occult Blood Gold Gel Stripe, Van Ed Chapman Biological Engineering Company), which was performed independently at the laboratory of the Zhongnan Hospital of Wuhan University. This study was approved by the Ethical Committee of our university, and all patients gave informed consent for their participation.

Carcinomas were classified according to the Union for International Cancer Control (UICC) TNM classification system. Advanced adenoma was defined as an adenoma of least 10 mm in diameter, with high-grade dysplasia, with villous or tubulovillous histological characteristics, or any combination of the three [10]. The left colon was defined as the region from the rectum to the splenic flexure, and the right colon was defined as being above the transverse colon.

DNA extraction

All stool samples were coded before being randomized and processed. Genomic DNA was extracted from clinical fecal samples (200 mg) with the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer´s instructions.

Bisulfite and modification treatments

Bisulfite genomic DNA modification and purification, which converts all unmethylated cytosines to uracils, was performed as reported previously [11]. For verification of the quality of the extracted DNA, the human β-actin gene must be amplified by PCR. Each PCR reaction mix consisted of a total volume of 25 μl containing 10× PCR buffer (Thermo,

United States), 12 μl PCR-Mix (Topsun Group, China), a 1 μM concentration of each of the primers β-actin-F (TGGTGATGGAGGAGGCTCAGCAAGT) and β-actin-R (AGCCAATGGGACCTGCTCCTCCCTTGA), and 5 to 20 ng fecal DNA. The PCR samples were subjected to 94℃ for 3 min, followed by 35 cycles at 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 1 min, and finally a 10 min extension at 72℃. PCR products were analyzed by 3% agarose gel electrophoresis.

Methylation-specific PCR (MSP) analysis

After the bisulfite and modification treatment, the target genes were amplified with primers for the methylated and unmethylated genes. The primer sequences are listed in Table 2.

The procedures of MSP are as follows: 2 μl modified DNA was amplified in a total volume of 25 μl containing 2.5 μl of 10×PCR buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 0.5 μmol/L of each primer, and 1.0 unit of Taq polymerase. Amplification conditions included a hot start at 95℃ for 5 min, 40 cycles at 95℃ for 45 s, annealing temperature of each gene for 45 s, 72℃ for 45 s, and a final 8 min extension step at 72℃. Products were visualized by 3% agarose gel electrophoresis with ultraviolet illumination using a gel imaging analyzing system (Genesnap Syngene, China). Methylation pattern analysis was based on the distribution of visible bands.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software version 17.0. Associations between the discrete variables were assessed using the two-sided Fisher's exact test or Pearson's chi square tests. A P-value less than 0.05 was regarded as statistically significant.

RESULTS

Hypermethylation of SFRP2 and WIF-1 genes in fecal DNA

Hypermethylation of SFRP2 and WIF-1 in fecal samples was detected in 56.3% (27/48) and 60.4% (29/48) of 48 CRC, respectively, and 81.3% (39/48) of CRC samples had at least one hypermethylated gene detected. The frequency of the aberrant methylation of SFRP2 and WIF-1 were 51.4% (18/35) and 45.7% (16/35) in colorectal adenoma samples, and 65.7%

(23/35) of adenoma samples had at least one hypermethylated gene. Hypermethylation of SFRP2 and WIF-1 in fecal samples was detected in 12.5% (4/32) and 18.7% (6/32) of 32 hyperplastic polyp samples, respectively, and 25% (8/32) of the hyperplastic polyp samples had at least one hypermethylated gene. In 30 normal fecal samples, one case exhibited a band representative of methylated WIF-1 (Figure, Table 3).

The hypermethylation of SFRP2 and WIF-1 genes was significantly different between the carcinoma and normal control groups (both $P<0.001$), the carcinoma and hyperplastic polyp samples (both $P<0.001$), the adenoma group vs. normal controls (both $P<0.001$), between the adenoma and hyperplastic polyp groups ($P<0.001$ for SFRP2, $P=0.005$ for WIF-1), and between the hyperplastic polyp group and normal controls ($P=0.045$ for SFRP2, but $P=0.055$ for WIF-1). No significant difference was found between carcinoma and adenoma ($P=0.663$ for SFRP2, and $P=0.184$ for WIF-1).

Hypermethylated SFRP2 and WIF-1 were detected in 71.4% (5/7) and 57.1% (4/7) of fecal samples from patients with Dukes A stage of CRC, and 60.0% (9/15) and 53.3% (8/15) of those with advanced adenoma, respectively. The frequency of combined SFRP2 and WIF-1 hypermethylation was higher than when detected singly. The frequency of hypermethylated SFRP2 and WIF-1 in fecal samples reached 80.0% for advanced adenoma, which is higher than that of non-advanced adenoma (55%). Additionally, 81.8% (18/22) of Dukes A stage of CRC and advanced adenoma patients had at least one hypermethylated gene detected in the fecal samples.

Moreover, no significant associations were observed between the hypermethylation of SFRP2 and WIF-1 genes and other clinicopathological features, including gender, age, tumor location and TNM stage, lymph node status and distant metastasis, etc. (Table 1).

Hypermethylated genes in fecal DNA and FOBT

There were 83 colorectal tumors (48 CRC and 35 adenomas) and there was a distinct difference ($P<0.001$) in the efficiency of diagnosing colorectal tumors when using the fecal hypermethylated SFRP2 and WIF-1 genes (74.7%, 62/83) vs. FOBT (43.4%, 36/83), as well as in tumors classified as Dukes A of CRC ($P=0.031$) and adenoma ($P<0.001$). There was no significant difference when comparing diagnosis by hypermethylation or FOBT in Dukes B-D of CRC ($P>0.05$), samples of hyperplastic polyps and normal subjects ($P>0.05$) (Table 4).

DISCUSSION

Sporadic colon cancer development is a multistep developmental process with the accumulation of genetic and epigenetic alterations that transform colonic epithelial cells into colon adenocarcinoma cells [12]. Recently, the epigenetic silencing of tumor suppressor genes has been increasingly recognized as an important mechanism in tumorigenesis, and the detection of hypermethylated genes in patient stools has emerged as a biologically rational and user-friendly strategy for early CRC [13][14, 15].

Aberrant activation of the Wnt signaling pathway is associated with a variety of human cancers, particularly colorectal cancer, as well as the physiological function of adult organisms. Aberrant promoter hypermethylation of Wnt antagonist genes frequently occurs in the early stages of the pathogenesis of human cancers, and up to 90% of colorectal cancers result in aberrant activation of Wnt signaling [16]. Previous studies have identified that SFRP2 and WIF-1, both secreted Wnt antagonists, are silenced epigenetically [8, 17].

Our results here and in our previous study [9] have shown that the promoters of SFRP2 and WIF-1 genes are frequently hypermethylated in patients with colorectal tumors and can be detected in their fecal samples. The results showed that SFRP2 and WIF-1 were hypermethylated in 56.3% and 60.4% of CRC, 51.4% and 45.7% of adenomas, and 12.5% and 18.7% of hyperplastic polyp cases, respectively, thereby indicating that the hypermethylation of SFRP2 and WIF-1 occurs in hyperplastic polyps and becomes more frequent in adenomas and then finally in colorectal cancers. Our results showed that hypermethylated SFRP2 and WIF-1 genes in fecal samples had reached 81.8% (18/22) in advanced adenomas and Dukes A of CRC patients. Therefore, examination of the SFRP2 and WIF-1 hypermethylation status may provide useful information for detecting the early onset of CRC, indicating that the hypermethylation of SFRP2 and WIF-1 is closely linked to the initiation and progression of CRC carcinogenesis.

Historically, colorectal adenoma has been recognized as the most important precancerous lesion occurring prior to fulminant CRC. It is estimated that fifty percent of individuals will develop adenomas in their lifetime, but only six percent will progress into CRC [5, 18]. Therefore, most adenomas do not progress to cancer. We showed that the frequency of hypermethylated SFRP2 was higher in advanced adenoma than in

non-advanced adenoma. Moreover, the difference between the methylation states of genes in CRC and advanced adenoma was not significant ($P>0.05$), while the difference between epigenetic changes in CRC and non-advanced adenoma was significant ($P<0.05$). This suggests that removing advanced adenomas rather than the non-advanced ones is important in the screening for early CRC. Additionally, removing advanced adenomas could be used complementary with colonoscopy to reduce the high cost of surveillance endoscopy and could improve patient compliance with surveillance, which is currently poor, even among high-risk patients [19][20, 21].

The diagnostic sensitivity attained by screening for combined SFPR2 and WIF-1 hypermethylation is higher than screening for a single hypermethylated gene. The clinical sensitivities for detecting CRC, advanced adenoma and hyperplastic polyps reached 81.3%, 80.0% and 25.0%, respectively, and the specificity was 96.7%. In addition, there was a trend, although not statistically significant, of more frequent SFRP2 and WIF-1 hypermethylation among the adenomas accompanied with epithelial dysplasia. Hypermethylation of the WIF-1 gene was found in one normal subject endoscopically, which was likely attributed to the presence of premalignant lesions, such as aberrant crypt foci (ACF).

To further address the clinical relevance of hypermethylated SFRP2 and WIF-1 in CRC, our data suggested that assaying for hypermethylated genes works equally well for detecting proximal and distal colorectal tumors, in agreement with a previous study illustrating that the detection of hypermethylated genes was not influenced by the site of the neoplasm [22]. Considering the proportional rise in incidence of CRC and precancerous lesions in the proximal colon, measuring DNA hypermethylation in fecal samples plays an important role in the detection of proximal neoplasms [23, 24]. Although determinate studies are needed, our results suggest the potential usefulness of assaying the hypermethylation of Wnt antagonist genes in fecal samples for the detection of early CRC.

It is known that the majority of sporadic colorectal dysplasias and malignancies require the inactivation of the APC gene, promoting β-catenin mediated transcription. Therefore, in the absence of epigenetic silencing, APC mutation and the consequent β-catenin activity would lead to Wnt antagonist induction in a negative feedback loop, starving the tumor cell of Wnt ligand [25]. Because the epigenetic silencing of Wnt antagonists in genes occur in early sporadic colorectal tumorigenesis, the activation of Wnt

signaling may be necessary for survival of the early tumor [17]. In addition, the restoration of SFRPs and WIF-1 function in CRC cells attenuates Wnt/β-catenin signaling, resulting in a significant inhibition of colony formation and cell proliferation even in the presence of downstream mutations in APC or β-catenin. The hypermethylation of SFRP2 and WIF-1 has been detected in more than 90% and 80%, respectively, in primary CRC [26]. As the activation of Wnt pathway in colorectal tumors has been observed at high frequencies, and considering the important role it plays in both initiation and progression of CRC, the targeted inhibition of the Wnt signaling pathway provides a rational and promising approach for the screening of early CRC.

Colonocytes from the surface of the colonic mucosa are continuously shed into the lumen of the colon and excreted as a normal component of stool. These cells are shed from the lower crypts at a rate of at least 10^{10} cells per day, each with a lifespan of 3-4 days, and within four days, the entire colonic mucosa is renewed[27][28]. Current advances in colon cytobiology have led to the understanding that exfoliation is triggered by apoptosis in the normal colon, while in malignant lesions, genetic and/or epigenetic changes prevent this destruction of colonocytes, allowing them to survive within the stool [29]. Additionally, the number of colonocytes exfoliated from malignant lesions is 4- to 5-fold greater than from normal tissue [28], so it could be simple and effective to collect fecal samples containing colonic DNA for the detection of early CRC.

Fecal occult blood testing (FOBT), especially immunochemical FOBT (iFOBT), is the most widely utilized procedure for a non-invasive CRC screening method. Although FOBT is confirmed to reduce the mortality of CRC, the test has little impact on the incidence of CRC because of the low level of sensitivity to detect precursor lesions [30]. Stool DNA testing has emerged as a biologically rational and user-friendly strategy for the non-invasive detection of both CRC and precursor lesions [10].

In our results, the detection sensitivity for hypermethylated Wnt antagonist genes represents an apparent advantage to FOBT in the detection of CRC and colorectal adenoma, especially in the early cancer and advanced adenoma stages. This shows that measuring the hypermethylation of Wnt antagonist genes in fecal samples was superior to FOBT for the detection of colorectal tumors. This detection advantage may contribute to the apparently continuous exfoliation of cells from colorectal neoplasms, while occult bleeding is intermittent [31][32].

In summary, the detection of tumor-derived DNA alterations in the stool is an intriguing new approach with a high potential for the non-invasive detection of CRC. Our results demonstrate that the hypermethylation of SFRP2 and WIF-1 in fecal samples shows promise for the accurate detection of CRC. However, a large number of studies are required to further accurately confirm the role of hypermethylation SFRP2 and WIF-1 for the early CRC diagnosis.

COMMENTS

Background

Colorectal cancer (CRC) is the third-most common malignancy and the second leading cause of cancer-related deaths in western countries. The diagnosis of CRC in early stages has great importance for reducing CRC mortality. Although significant advances have been achieved in diagnostic technologies, the currently available modalities for diagnosing CRC remain suboptimal.

Research frontiers

DNA hypermethylation can result in the transcriptional silencing of tumor suppressor genes and plays an important role in the initiation and progression of sporadic CRC. Detection of hypermethylation of Wnt antagonist genes in human stool DNA might provide a novel and promising strategy for early CRC diagnosis.

Innovations and breakthroughs

Methylation-specific PCR (MSP) was performed to analyze the promoter hypermethylation status of the SFRP2 and WIF-1 genes in a blinded fashion in stool samples from patients with CRC and colorectal benign diseases. Furthermore, for the diagnosis of early CRC, we compared the sensitivity of assaying for the hypermethylated SFRP2 and WIF-1 genes to the standard fecal occult blood test (FOBT) in matched stool samples from the same patients.

Applications

Our study demonstrated initially that hypermethylated SFRP2 and WIF-1 genes in stool are promising and non-invasive sensitive markers for screening early CRC.

Terminology

Wnt signaling pathway: Signals are transduced through binding of Wnt-ligands to the Frizzled (Fz) family of seven-pass transmembrane receptors by competition for binding to Wnt-ligands or by the direct formation of non-functional complexes with Fz receptors. Wnt-antagonists: Wnt antagonists regulate the upstream signaling of the Wnt pathway outside of the cell, which are divided into two functional classes. The first functional class is the SFRPs (secreted Frizzled-related proteins), which include the SFRP family (SFRP1-5), WIF-1 (Wnt inhibitory factor-1) and Cerberus, which bind directly to Wnts. The second functional class is the Dickkopf (Dkk) class that binds to Wnts indirectly.

Peer review

This paper studied the use of hypermethylation of SFRP2 and WIF-1 promoter as a novel and promising approach for early CRC diagnosis. The authors showed that there exists a high prevalence of SFRP2 and WIF-1 hypermethylation in the stool DNA of colorectal neoplasms. There also appeared to be a significant difference in the sensitivity for diagnosing early CRC between the hypermethylation of SFRP2 and WIF-1 and FOBT. They conclude that the hypermethylation of SFRP2 and WIF-1 may be a sensitive diagnostic test for colorectal neoplasms.

REFERENCES

1 **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; 127(12): 2893-2917. [PMID: 21351269 DOI: 10.1002/ijc.25516]

2 **The gastroenterology of the Chinese medical association**. A consensus of the early detection, treatment, and the comprehensive prevention of colorectal cancer in China. *The Chinese Journal of Digestion* 2012; 32(1): 1-10. [DOI: 10.3760/cma.j.issn.0254-1432.2012.01.001]

3 **Chen WD**, Han ZJ, Skoletsky J, Olson J, Sah J, Myeroff L, Platzer P, Lu S, Dawson D, Willis J, Pretlow TP, Lutterbaugh J, Kasturi L, Willson JK, Rao JS, Shuber A, Markowitz SD. Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. *J Natl Cancer Inst* 2005; 97(15): 1124-1132. [PMID: 16077070 DOI: 10.1093/jnci/dji204]

4 **Bosch LJ**, Carvalho B, Fijneman RJ, Jimenez CR, Pinedo HM, van Engeland M, Meijer

GA. Molecular tests for colorectal cancer screening. *Clin Colorectal Cancer* 2011; 10(1): 8-23. [PMID: 21609931 DOI: 10.3816/CCC.2011. n.002]

5 **Jemal A**, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008; 58(2): 71-96. [PMID: 18287387 DOI: 10.3322/CA.2007.0010]

6 **Kohler BA**, Ward E, McCarthy BJ, Schymura MJ, Ries LA, Eheman C, Jemal A, Anderson RN, Ajani UA, Edwards BK. Annual report to the nation on the status of cancer, 1975-2007, featuring tumors of the brain and other nervous system. *J Natl Cancer Inst* 2011; 103(9): 714-736. [PMID: 21454908 DOI: 10.1093/jnci/djr077]

7 **Suzuki H**, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG, Baylin SB. A genomic screen for genes pregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet* 2002; 31(2): 141-149. [PMID: 11992124 DOI: 10.1038/ng892]

8 **Suzuki H**, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG, Baylin SB. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 2004; 36(4): 417-422. [PMID: 15034581 DOI: 10.1038/ng1330]

9 **Qi J**, Zhu YQ, Luo J, Tao WH. Hypermethylation and expression regulation of secreted frizzled-related protein genes in colorectal tumor. *World J Gastroenterol* 2006; 12(44): 7113-7117.[PMID: 17131472]

10 **Qi J**, Zhu YQ. Targeting the most upstream site of Wnt signaling pathway provides a strategic advantage for therapy in colorectal cancer. *Curr Drug Targets* 2008; 9(7): 548-557. [PMID: 18673240]

11 **Ahlquist DA**. Molecular detection of colorectal neoplasia. *Gastroenterology* 2010; 138(6): 2127-2139. [PMID: 20420950 DOI: 10.1053/j. gastro.2010.01.055]

12 **Herman JG**, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996 1996-09-03; 93(18): 9821-9826. [PMID: 8790415]

13 **Grady WM**, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008; 135(4): 1079-1099. [PMID: 18773902 DOI: 10.1053/j.gastro.2008.07.076]

14 **Lao VV**, Grady WM. Epigenetics and colorectal cancer. *Nat Rev Gastroenterol Hepatol* 2011; 8(12): 686-700. [PMID: 22009203 DOI: 10.1038/nrgastro.2011.173]

15 **Berger BM**, Ahlquist DA. Stool DNA screening for colorectal neoplasia: biological and technical basis for high detection rates. *Pathology* 2012; 44(2): 80-88. [PMID: 22198259 DOI: 10.1097/PAT.0b013e3283502fdf]

16 **Zou H**, Taylor WR, Harrington JJ, Hussain FT, Cao X, Loprinzi CL, Levine TR, Rex DK, Ahnen D, Knigge KL, Lance P, Jiang X, Smith DI, Ahlquist DA. High detection rates of colorectal neoplasia by stool DNA testing with a novel digital melt curve assay. *Gastroenterology* 2009; 136(2): 459-470. [PMID: 19026650 DOI: 10.1053/j.gastro.2008.10.023]

17 **Suzuki H**, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG, Baylin SB. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 2004; 36(4): 417-422. [PMID: 15034581 DOI: 10.1038/ng1330]

18 **He B**, Reguart N, You L, Mazieres J, Xu Z, Lee AY, Mikami I, McCormick F, Jablons DM. Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations. *Oncogene* 2005; 24(18): 3054-3058. [PMID: 15735684 DOI: 10.1038/sj.onc.1208511]

19 **Strul H**, Kariv R, Leshno M, Halak A, Jakubowicz M, Santo M, Umansky M, Shirin H, Degani Y, Revivo M, Halpern Z, Arber N. The prevalence rate and anatomic location of colorectal adenoma and cancer detected by colonoscopy in average-risk individuals aged 40-80 years. *Am J Gastroenterol* 2006; 101(2): 255-262. [PMID: 16454827 DOI: 10.1111/j.1572-0241.2006.00430.x]

20 **Rubenstein JH**, Waljee AK, Jeter JM, Velayos FS, Ladabaum U, Higgins PD. Cost effectiveness of ulcerative colitis surveillance in the setting of 5-aminosalicylates. *Am J Gastroenterol* 2009; 104(9): 2222-2232. [PMID: 19491824 DOI: 10.1038/ajg.2009.264]

21 **Velayos FS**, Liu L, Lewis JD, Allison JE, Flowers N, Hutfless S, Abramson O, Perry GS, Herrinton LJ. Prevalence of colorectal cancer surveillance for ulcerative colitis in an integrated health care delivery system. *Gastroenterology* 2010; 139(5): 1511-1518. [PMID: 20659470 DOI: 10.1053/j.gastro.2010.07.039]

22 **Vienne A**, Simon T, Cosnes J, Baudry C, Bouhnik Y, Soule JC, Chaussade S, Marteau P, Jian R, Delchier JC, Coffin B, Admane H, Carrat F, Drouet E, Beaugerie L. Low prevalence of colonoscopic surveillance of inflammatory bowel disease patients with longstanding extensive colitis: a clinical practice survey nested in the CESAME cohort. *Aliment Pharmacol Ther* 2011; 34(2): 188-195. [PMID: 21615760 DOI:

10.1111/j.1365-2036.2011.04711.x]

23 **Ahlquist DA**, Zou H, Domanico M, Mahoney DW, Yab TC, Taylor WR, Butz ML, Thibodeau SN, Rabeneck L, Paszat LF, Kinzler KW, Vogelstein B, Bjerregaard NC, Laurberg S, Sorensen HT, Berger BM, Lidgard GP. Next-generation stool DNA test accurately detects colorectal cancer and large adenomas. *Gastroenterology* 2012; 142(2): 248-256, e25-e26. [PMID: 22062357 DOI: 10.1053/j.gastro.2011.10.031]

24 **Gupta AK**, Melton LR, Petersen GM, Timmons LJ, Vege SS, Harmsen WS, Diehl NN, Zinsmeister AR, Ahlquist DA. Changing trends in the incidence, stage, survival, and screen-detection of colorectal cancer: a population-based study. *Clin Gastroenterol Hepatol* 2005; 3(2): 150-158. [PMID: 15704049]

25 **Park SY**, Kim BC, Shin SJ, Lee SK, Kim TI, Kim WH. Proximal shift in the distribution of adenomatous polyps in Korea over the past ten years. *Hepatogastroenterology* 2009; 56(91-92): 677-681. [PMID: 19621679]

26 **Caldwell GM**, Jones CE, Soon Y, Warrack R, Morton DG, Matthews GM. Reorganisation of Wnt-response pathways in colorectal tumorigenesis. *Br J Cancer* 2008; 98(8): 1437-1442. [PMID: 18414471 DOI: 10.1038/sj.bjc.6604327]

27 **Itzkowitz S**, Brand R, Jandorf L, Durkee K, Millholland J, Rabeneck L, Schroy PR, Sontag S, Johnson D, Markowitz S, Paszat L, Berger BM. A simplified, noninvasive stool DNA test for colorectal cancer detection. *Am J Gastroenterol* 2008; 103(11): 2862-2870. [PMID: 18759824 DOI: 10.1111/j.1572- 0241.2008.02088.x]

28 **Davies RJ**, Miller R, Coleman N. Colorectal cancer screening: prospects for molecular stool analysis. *Nat Rev Cancer* 2005; 5(3): 199-209. [PMID: 15738983 DOI: 10.1038/nrc1545]

29 **Nair P**, Lagerholm S, Dutta S, Shami S, Davis K, Ma S, Malayeri M. Coprocytobiology: on the nature of cellular elements from stools in the pathophysiology of colonic disease. *J Clin Gastroenterol* 2003; 36(5 Suppl): S84-S93, S94-S96. [PMID: 12702972]

30 **Heresbach D**, Manfredi S, D'Halluin PN, Bretagne JF, Branger B. Review in depth and meta-analysis of controlled trials on colorectal cancer screening by faecal occult blood test. *Eur J Gastroenterol Hepatol* 2006; 18(4): 427-433. [PMID: 16538116]

31 **Ahlquist DA**, Harrington JJ, Burgart LJ, Roche PC. Morphometric analysis of the "mucocellular layer" overlying colorectal cancer and normal mucosa: relevance to exfoliation and stool screening. *Hum Pathol* 2000; 31(1): 51-57. [PMID: 10665913]

32 **Ahlquist DA**, Sargent DJ, Loprinzi CL, Levin TR, Rex DK, Ahnen DJ, Knigge K, Lance

MP, Burgart LJ, Hamilton SR, Allison JE, Lawson MJ, Devens ME, Harrington JJ, Hillman SL. Stool DNA and occult blood testing for screen detection of colorectal neoplasia. *Ann Intern Med* 2008; 149(7): 441-450, W81. [PMID: 18838724]

Table 1 Correlation of the methylation status of fecal SFRP2 and WIF-1 genes with patients' clinicopathological parameters

rameter	Case (n)	WIF-1		SFRP2		WIF-1+ SFRP2		sensitivity	specificity
		methlation (%)	P value	methlation (%)	P value	methlation (%)	P value		
C	48	27(56.3%)	0.000*	29 （60.4%）	0.000*	39 （81.3%）	0.000*	81.3%	
nder									
Male	24	17(70.8%)	0.140	14(58.3%)	0.771				
Female	24	12(29.2%)		13(54.2%)					
e(year)									
>55	32	21(65.6%)	0.297	18(56.3%)	1.000				
≤55	16	8(50%)		9(56.3%)					
mor ation									
eft colon	32	21(65.6%)	0.267	18(56.3%)	1.000				
ght colon	16	8(50%)		9(56.3%)					
M stage									
A～B	27	18(66.7%)	0.315	15(55.6%)	0.912				
C～D	21	11(52.4%)		12(57.1%)					
mph de tastasis									
Positive	21	11(52.4%)	0.315	12(57.1%)	0.912				
Negative	27	18(66.7%)		15(55.6%)					
tant tastasis									
Positive	7	4(57.1%)	0.848	4(57.1%)	0.959				
Negative	41	25(61.0%)		23(56.1%)					
enoma	35	18(51.4%)	0.000*	16(45.7%)	0.000*	23(65.7%)	0.000*	65.7%	
Advanced	15								
n-advanc ed	20								
perplasti olyp	32	4(12.5%)	0.045*	6(18.7%)	0.055*	8(25.0%)	0.016*	25.0%	96.7%
rmal	30	0(0%)		1(3.3%)		1(3.3%)			

*Represents the P-value vs. normal subjects. Sensitivity represents the ability of the DNA methylation test to detect the presence of at least one hypermethylated gene.

Table 2 SFRP2 and WIF-1 gene primer sequences for methylation-specific PCR assays

genes	primer (5' → 3')	Product size (bp)	Annealing temperature (℃)
M:*SFRP2*	GGGTCGGAGTTTTTCGGAGTTGCGC	138 bp	62℃
	CCGCTCTCTTCGCTAAATACGACTCG		(38)
U:*SFRP2*	TTTTGGGTTGGAGTTTTTTGGAGTTGTGT	145 bp	52℃
	AACCCACTCTCTTCACTAAATACAACTCA		(38)
M:*WIF-1*	GGGCGTTTTATTGGGCGTAT	197bp	58℃
	AAACCAACAATCAACGAAC		(38)
U:*WIF-1*	GGGTGTTTTATTGGGTGTAT	198bp	55℃
	AAACCAACAATCAACAAAAC		(38)

Table 3 The hypermethylation status of SFRP2 and WIF-1 in fecal DNA samples

Group	Case	Hypermethylated genes		
		SFRP2 (%)	WIF-1 (%)	SFRP2+ WIF-1 (%)
CRC	48	27(56.3%)	29（60.4%）	39（81.3%）
Dukes A-B	27	15(55.6%)	18(66.7%)	22(81.5%)
Dukes A	7	5(71.4%)	4(57.1%)	6(85.7%)
Dukes B	20	10(50.0%)	14(70.0%)	16(80.0%)
Dukes C-D	21	12(57.1%)	11(52.4%)	17(81.0%)
Dukes C	14	8(57.1%)	7(50.0%)	10(71.4%)
Dukes D	7	4(57.1%)	4(57.1%)	7(100%)
Adenoma	35	18(51.4%)	16(45.7%)	23(65.7%)
Advanced	15	9(60.0%)	8(53.3%)	12(80.0%)
Non-advanced	20	8(40.0%)	7(35.0%)	11(55.0%)
Hyperplastic polyp	32	4(12.5%)	6(18.7%)	8(25.0%)
Normal control	30	0(0%)	1(3.3%)	1(3.3%)

Table 4 Hypermethylated SFRP2 and WIF-1 genes and FOBT for the detection of colorectal tumors

group	N	Hypermethylated	FOBT	P values

		SFRP2 and WIF-1		
CRC	48	39(81.3%)	27(56.3%)	0.008
Dukes A-B	27	22(81.5%)	14(51.9%)	0.021
Dukes A	7	6(85.7%)	2(28.5%)	0.031
Dukes B	20	16(80.0%)	12(60.0%)	0.168
Dukes C-D	21	17(81.0%)	13(61.9%)	0.17
Dukes C	14	10(71.4%)	8(57.1%)	0.430
Dukes D	7	7(100%)	5(71.4%)	0.127
Adenoma	35	23(65.7%)	9(25.7%)	<0.001
Advanced	15	12(80.0%)	5(33.3%)	0.010
non-advanced	20	11(55.0%)	4(20.0%)	0.022
Hyperplastic polyp	32	8(25.0%)	5(15.6%)	0.351
Normal control	30	1(3.3%)	1(3.3%)	1.000

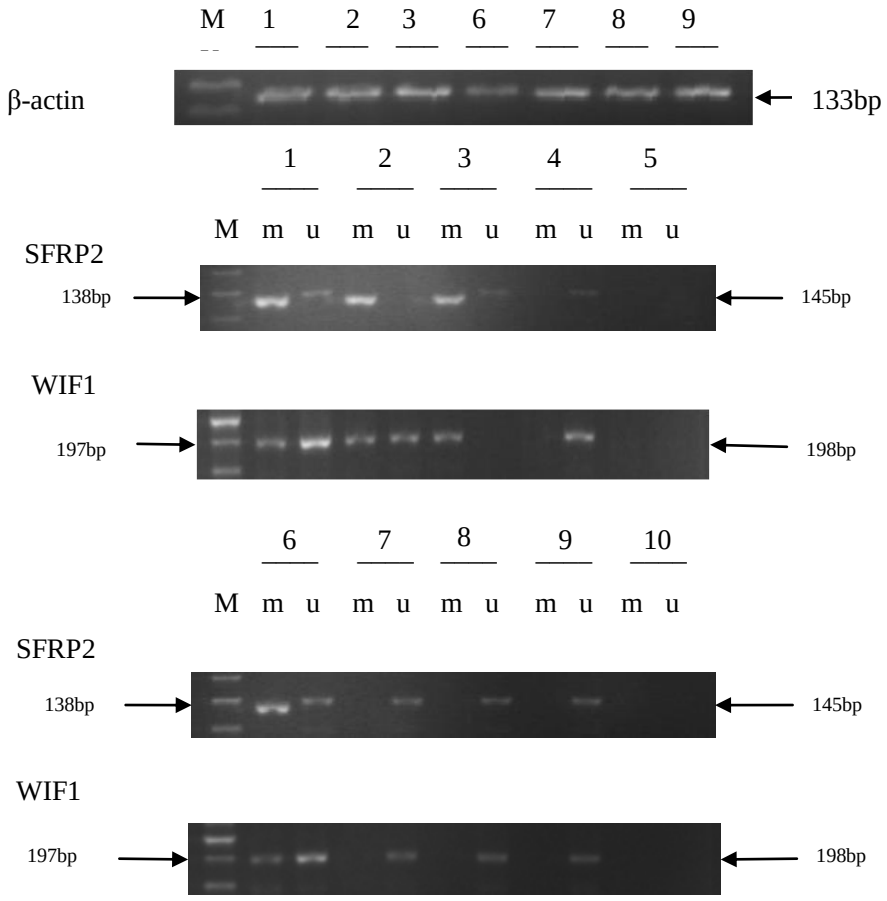


Figure Methylation status of SFRP2 and WIF-1 genes in clinical stool samples. 1-3: colorectal cancers; 6-8: polyps (colorectal adenomas or hyperplastic polyps); 4, 9: normal

controls; 5, 10: blank controls; M: 50 bp DNA marker; m: methylated bands; u: unmethylated bands.