JBUON 2021; 26(4): 1239-1245
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

# Highly sensitive fecal DNA testing of NDRG4 12b methylation is a promising marker for detection of colorectal precancerosis

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## Summary

***Purpose:*** *The purpose of this study was to research and validate techniques for extracting DNA from human genomes, explore the sensitivity and specificity of known nucleic acid markers of intestinal malignancy in Chinese patients with early colorectal cancer. We also tried to find adenoma-specific biomarkers in human DNA in feces.*

***Methods:*** *We compared the ability of fecal DNA testing, Fecal Occult Blood Testing (FOBT) and serum tumor markers to diagnose different types of polyps, and DNA testing was significantly superior to the other two methods. We also found a dominant expression of NDRG12b methylation in multi-target DNA testing, which may be a promising marker for detection of colorectal precancerosis.*

***Results:*** *The sensitivity of NDRG4 12b methylation was 85.7% for advanced adenomatous polyp (AP), and 62.6% for non-advanced AP, respectively, with specificity of 70.8%. The diagnostic efficacy of NDRG4 12b methylation for detecting advanced AP was significantly higher than FOBT (sesitivity: 85.7% vs. 42.9%, p<0.05). The receiver operating characteristics (ROC) curve for NDRG4 12b methylation in detecting AP showed a relatively high area under the curve (AUC=0.807).*

***Conclusions:*** *Our results indicate that highly sensitive fecal DNA testing of NDRG4 12b methylation is a promising marker for detection of colorectal precancerosis, especially in detecting adenomatous polyp.*

***Key words:*** *fecal DNA testing, methylation, fecal occult blood testing, colorectal precancerosis, sensitivity*

## Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies in the world, with the third highest incidence and second highest mortality worldwide [1]. According to latest researches fecal stool contains a small amount of nucleic acid derived from human intestinal epidermal cells. Multitarget detection of these nucleic acids can effectively predict malignancies. A study indicates that the fecal immunochemical test (FIT) sensitivity is maximised to 97.0% at the lowest cut-off (2 μg/g) and a negative FIT result at this cut-off can effectively rule out CRC and a positive FIT result is better than symptoms to select patients for urgent investigations [2], while FIT has limitations in detecting early-stage CRC and advanced adenoma. Recently, DNA methylation testing has emerged as a new molecular approach [3]. The ACG's latest CRC screening guidelines recommend multitarget stool DNA testing every three years and colonoscopy every five to ten years [4]. Fecal DNA tests have shown that the technique can prospectively screen for CRC. Based on the results of

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Received: 26/05/2021; Accepted: 08/07/2021

a clinical trial involving 9989 CRC patients from more than 90 different regions in two countries, the United States and Canada, the results indicate that the sensitivity for detecting CRC was 92.3% with DNA testing and 73.8% with FIT, the rate of detection of polyps with high-grade dysplasia was 69.2% with DNA testing and 46.2% with FIT [5]. The technique was approved by the FDA for CRC screening in 2014 and has been listed in the NCCN guidelines for CRC screening and the National Cancer Prevention Program.

Gene methylation [6] plays an important role in the process of intestinal epithelial malignancy. The characteristics of methylation at the tissue level of CRC have been recognized in TCGA and other studies, but the characteristics of methylation in adenoma remain to be studied. Peripheral blood or fecal sampling provide great opportunities for CRC screening and diagnosis. However, there are realistic barriers to identifying these CRC-specific methylation features in these biological samples. We now have a technology that makes it easy to extract total human DNA from feces. The DNA methylation biomarkers BMP3, NDRG4 and SDC2 have been extensively studied [7-16]. In this study we explored the use of feces as an antecedent for the examination of intestinal epithelial malignancy.

## Methods

*Study population*

We enrolled 238 individuals from December 2018 through November 2019 at the First Affiliated Hospital of Nanjing Medical University, of whom 199 had results that could be fully evaluated (Figure 1). The target population was persons aged between 40 and 75 years who had physical examinations, with changes in stool habits, and with a family history of CRC. We excluded participants who were menstruating at the time of fecal sampling, had diarrhea at the time of feces collection, without formed feces, had other tumors in their personal history, had previous CRC surgery history, diagnosed with inflammatory bowel disease, diagnosed with esophageal and gastric bleeding symptoms, diagnosed with hemorrhoids, and fecal sampling was during the bleeding period. This clinical study was approved by the Institutional Review Board of the First Affiliated Hospital of Nanjing Medical University (No. 2019-SR-311).

*Clinical procedures*

A medical history, physical examination and laboratory examinations were performed after the signing of the informed consent form. In this study, according to the 2013 Adult Weight Determination, weight was classified according to body mass index (BMI) (kg/m$^2$): underweight (<18.5), normal weight (18.5~), overweight (24.0~) and obese (≥28.0). All participants were required to provide a stool specimen and undergo screening colonoscopy. The right colon was considered to include the splenic flexure and all segments proximal to it, an insertion depth of more than 60 cm, the left colon was considered to include all other segments, an insertion depth of 60 cm or less.

*DNA isolatin and bisulfite treatment*

Collected stool samples were weighted (200 mg) and centrifuged at 10,000 *g* for 10 min (Thermo Heraeus Multifuge X3R centrifuge, Thermo Scientific, Waltham, MA, USA), and 1 mL of the supernatant was transferred into a new centrifuge tube. Subsequently, 1 mL of adsorbent (TIANGEN, Beijing, China) was added to each sample and further centrifuged at 13,500 rpm for 3 min (Thermo Sorvall Micro 21R centrifuge, Thermo Scientific, Waltham, MA, USA), and 800 μL of the supernatant were then transferred into a new tube. Next, 200 μL of Lava-new buffer (TIANGEN, Beijing, China) and Protease K were added to each sample and incubated together

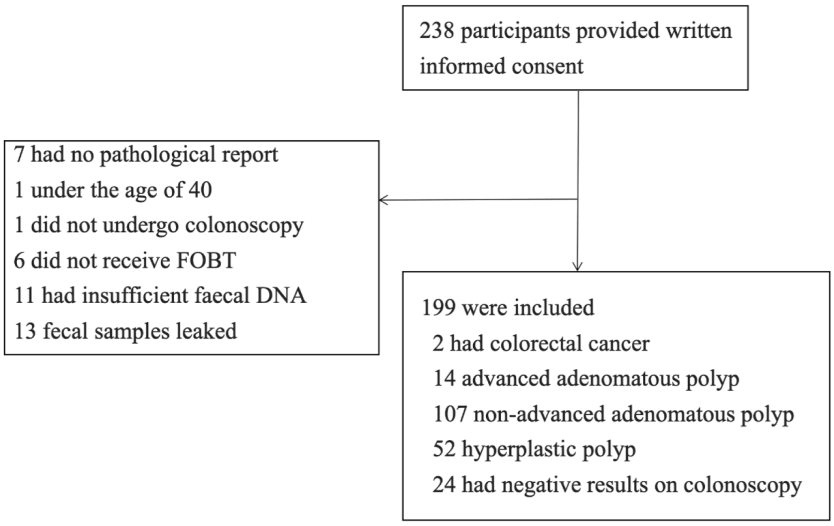


**Figure 1.** Patient enrollment and outcomes.

at 70°C for 15 min (Thermomixer, Thermo Scientific, Waltham, MA, USA). Each sample was added with 500 µL of chloroform (Sinopharm Chemical Reagent, Beijing, China) and centrifuged at 13,500 rpm for 10 min (Thermo Sorvall Micro 21R centrifuge, Thermo Scientific, Waltham, MA, USA). Subsequently, 900 µL of the supernatant were then transferred into a new tube and added with an equal volume of anhydrous ethanol (Sinopharm Chemical Reagent, Beijing, China). Solution and precipitation of each sample were added to a Spin Columns CB3 (TIANGEN, Beijing, China) and centrifuged at 13,500 rpm for 30 s (Thermo Sorvall Micro 21R centrifuge, Thermo Scientific, Waltham, MA, USA) to discard the solution. Next, 500 µL of GD buffer (TIANGEN, Beijing, China) and 600 µL of PW buffer (TIANGEN, Beijing, China) were added into the Spin Columns CB3 (TIANGEN, Beijing, China) to remove impurities, and were further centrifuged at 13,500 rpm for 30 s (Thermo Sorvall Micro 21R centrifuge, Thermo Scientific, Waltham, MA, USA) and dried. The stool DNA was finally eluted in 100 µL of TE buffer (TIANGEN, Beijing, China) and stored at -20°C until further use.

The stool DNA was chemically modified using an EZ DNA Methylation-GoldTM kit (ZYMO Research, Irvine, CA, USA) according to the manufacturer's instructions. Bisulfite-converted DNA was either used immediately for methylation analysis or stored at -20°C until further use.

*Methylation assays*

The methylation status in BMP3 and NDRG4 promoters were analyzed from TCGA database. Four regions were selected based on the significant difference between CRC tissues and benign tissues. They are named as BMP3, NDRG4 (NDRG4_12b, NDRG4_12m, NDRG4_34b). SDC2 regions were selected from the previous reports. Methylation assays were performed using KAPA PROBE FORCE qPCR Kits (KAPA Biosystems, Nutley, NJ, USA) in LightCycler® 480 Instrument II (Roche, Basel, Switzerland). A total of 20 µL reaction mixture consisting of 8.8 µL of bisulfite-converted stool DNA, 0.8 µL of methylation-specific antisense primers and 0.4 µL specific probe primers, and 10 µL of PROBE FORCE qPCR master (Roche, Basel, Switzerland) was prepared. Methylation-specific primers and probes were designed to bind to bisulfite-converted methylated DNA of the BMP3, NDRG4 (NDRG4_12b, NDRG4_12m, NDRG4_34b), and SDC2 genes. Locked nucleic acids (LNA) were used in the probes at the methylation sites to increase the specificities. Actin served as a reference gene to confirm PCR adequacy and quality of bisulfite-converted stool DNA. Thermal cycling conditions were as follows: 98°C for 3 min; 15 cycles at 95°C for 10 s and 65°C for 30 s; 35 cycles at 95°C for 10 s and 58°C for 30 s.

*Fecal occult blood testing (FOBT)*

FOBT was performed according to the manufacturer's instructions on the same stool sample used for the DNA test. Briefly, one drop of the peroxide catalyst was added into the reverse side of each window of the test cards, and a blue color reaction within 60 s was considered as a positive result.

*Statistics*

SPSS V.23.0 (IBM, Armonk, NY, USA) was used for retrospective data analyses. The area under receiver operating characteristic (ROC) curve (AUC) was calculated.

**Table 1.** Patient characteristics of different pathological classifications

| | *Advanced AP (n=14)* *n (%)* | *Nonadvanced AP (n=107)* *n (%)* | *HP (n=52)* *n (%)* | *Control (n=24)* *n (%)* | *p value* |
|---|---|---|---|---|---|
| Male sex | 10 (71.4) | 74 (69.2) | 38 (73.1) | 9 (37.5) | 0.014 |
| Age (years) | 61.5±8.2 | 58.5±8.9 | 59.7±8.7 | 57.6±7.9 | 0.321 |
| ≤65 | 8 (57.1) | 82 (76.6) | 38 (73.1) | 20 (83.3) | |
| >65 | 6 (42.9) | 25 (23.4) | 14 (26.9) | 4 (16.7) | |
| BMI | 24.4±2.2 | 24.6±3.0 | 23.9±3.0 | 24.6±2.5 | 0.664 |
| <18.5 | 0 (0) | 4 (3.7) | 2 (3.8) | 0 (0) | |
| 18.5~ | 6 (42.9) | 41 (38.3) | 25 (48.2) | 10 (41.7) | |
| 24.0~ | 7 (50.0) | 46 (43.0) | 23 (44.2) | 10 (41.7) | |
| ≥28.0 | 1 (7.1) | 16 (15.0) | 2 (3.8) | 4 (16.6) | |
| Smoking | 1 (7.1) | 35 (32.7) | 19 (36.5) | 2 (8.3) | 0.015 |
| Drinking | 2 (14.3) | 27 (25.2) | 15 (28.8) | 2 (8.3) | 0.191 |
| Hypertension | 8 (57.1) | 42 (39.1) | 18 (34.6) | 9 (37.5) | 0.507 |
| Diabetes | 2 (14.3) | 4 (3.7) | 3 (5.8) | 3 (12.5) | 0.141 |
| Family history of CRC | 0 (0) | 11 (10.3) | 4 (7.7) | 0 (0) | 0.348 |
| History of cancer disease | 1 (7.1) | 6 (5.6) | 1 (1.9) | 0 (0) | 0.415 |
| History of adenoma | 0 (0) | 2 (1.9) | 1 (1.9) | 0 (0) | 1.000 |
| Changes in stool habits | 1 (7.1) | 13 (12.1) | 4 (7.7) | 1 (4.2) | 0.722 |
| Bloody stool | 1 (7.1) | 1 (0.9) | 0 (0) | 1 (4.2) | 0.154 |

CRC: colorectal cancer, AP: adenomatous polyp, HP: hyperplastic polyp

Categorical variables were summarised as frequencies (%). To test for association between categorical variables, the Pearson $x^2$ or the Fisher exact test were used where appropriate. P value<0.05 was considered to be significant.

## Results

*Patient characteristics*

The baseline demographics, as well as the clinical characteristics of the study patients are summarized in Table 1. Overall, except for gender and smoking history, there were no significant differences between the four pathologic groups. Men were more likely to develop adenomatous polyp.

*DNA testing, FOBT and serum tumor markers*

As shown in Table 2, the sensitivity of SDC2, NDRG4 12b, NDRG4 34b and FOBT for advanced adenomatous polyp (AP) were 35.7%, 85.7%, 14.3% and 42.9%, respectively. Among the different detection methods we compared, NDRG 12b showed the highest sensitivity. The sensitivity of NDRG4 12b methylation was 85.7% for advanced AP, 62.6% for non-advanced AP, and 9.6% for HP. The sensitivity of NDRG 12b methylation in the diagnosis of AP was better than that of hyperplastic polyp (HP), especially of advanced AP (p<0.05). We selected CEA and CA19.9 as the serum tumor markers in the study. There were 7 patients with CEA positive results, including 5 non-advanced AP and 2 HP. There was only one patient with CA19.9 positive result and the colonoscopy was normal. Serum tumor markers did not show statistical significance in the pathologic diagnosis of polyps, and analysis did not carry out later.

We also performed a subgroup analysis of adenomatous polyps, as shown in Table 3. The sensitivity of NDRG4 12b, FOBT for AP with high-grade intraepithelial neoplasia (HGIN) was 85.7% and 42.9%, respectively. The NDRG4 12b methylation exhibited a significantly higher detection sensitivity in the AP with HGIN. The sensitivity of NDRG4 12b was 64.7% for AP with low-grade intraepithelial neoplasia (LGIN), which was significantly different from that for HP (p<0.05). In Figure 2, the

**Table 2.** Comparison of different detection methods

| | *Advanced AP (n=14)* *n (%)* | *Nonadvanced AP (n=107)* *n (%)* | *HP (n=52)* *n (%)* | *Control (n=24)* *n (%)* | *p value* |
|---|---|---|---|---|---|
| SDC2 | | | | | 0.005 |
| Positive results | 5 (35.7)[a] | 5 (4.7)[b] | 3 (5.8)[b] | 1 (4.2)[a,b] | |
| Negative results | 9 (64.3) | 102 (95.3) | 49 (94.2) | 23 (95.8) | |
| BMP3 | | | | | 0.073 |
| Positive results | 2 (14.3) | 17 (15.9) | 2 (3.8) | 1 (4.2) | |
| Negative results | 12 (85.7) | 90 (84.1) | 50 (96.2) | 23 (95.8) | |
| NDRG4 12b | | | | | 0.000 |
| Positive results | 12 (85.7)[a] | 67 (62.6)[a] | 5 (9.6)[b] | 7 (29.2)[b] | |
| Negative results | 2 (14.3) | 40 (37.4) | 47 (90.4) | 17 (70.8) | |
| NDRG4 12m | | | | | 0.445 |
| Positive results | 2 (14.3) | 8 (7.5) | 2 (3.8) | 1 (4.2) | |
| Negative results | 12 (85.7) | 99 (92.5) | 50 (96.2) | 23 (95.8) | |
| NDRG4 34b | | | | | 0.035 |
| Positive results | 2 (14.3) | 5 (4.7) | 1 (1.9) | 4 (16.7) | |
| Negative results | 12 (85.7) | 102 (95.3) | 51 (98.1) | 20 (83.3) | |
| FOBT | | | | | 0.030 |
| Positive results | 6 (42.9)[a] | 14 (13.1)[b] | 5 (9.6)[b] | 4 (16.7)[a,b] | |
| Negative results | 8 (57.1) | 93 (86.9) | 47 (90.4) | 20 (83.3) | |
| CEA | | | | | 1.000 |
| Positive results | 0 (0) | 5 (4.7) | 2 (3.8) | 0 (0) | |
| Negative results | 14 (100.0) | 102 (95.3) | 50 (96.2) | 24 (100.0) | |
| CA199 | | | | | 0.175 |
| Positive results | 0 (0) | 0 (0) | 0 (0) | 1 (4.2) | |
| Negative results | 14 (100.0) | 107 (100.0) | 52 (100.0) | 23 (95.8) | |

AP: adenomatous polyp; HP: hyperplastic polyp. The same marker letters indicate no statistical difference between the two groups.

ROC curve for NDRG4 12b methylation in detecting AP showed a relatively high area under the curve (AUC=0.807).

*Location and classification*

Among all detected genes, NDRG4 12b showed highest sensitivity. In Table 4, we studied the relationship between NDRG4 12b positive results and lesion location and classification. Polyps with NDRG4 12b positive results were more common in the left colon, type 0-Ip and type I were dominant in the Paris and Yamada classification, respectively. Among Paris classification, 80% of polyps with NDRG4 12b positive results were type 0-Ip, and the difference was statistically significant ($p<0.05$).

## Discussion

Our study investigated the diagnostic performances of DNA testing in detecting colorectal precancerosis and demonstrated an excellent diagnostic efficiency of NDRG4 12b methylation in detecting AP (AUC=0.807). The sensitivity of NDRG4

**Table 3.** Comparison of DNA testing and FOBT in subgroups

| | *AP with HGIN (n=14)* *n (%)* | *AP with LGIN (n=51)* *n (%)* | *HP (n=52)* *n (%)* | *Control (n=24)* *n (%)* | *p value* |
|---|---|---|---|---|---|
| SDC2 | | | | | 0.073 |
| Positive results | 4 (28.6) | 4 (7.8) | 3 (5.8) | 1 (4.2) | |
| Negative results | 10 (71.4) | 47 (92.2) | 49 (94.2) | 23 (95.8) | |
| BMP3 | | | | | 0.200 |
| Positive results | 2 (14.3) | 7 (13.7) | 2 (3.8) | 1 (4.2) | |
| Negative results | 12 (85.7) | 44 (86.3) | 50 (96.2) | 23 (95.8) | |
| NDRG4 12b | | | | | 0.000 |
| Positive results | 12 (85.7)[a] | 33 (64.7)[a] | 5 (9.6)[b] | 7 (29.2)[b] | |
| Negative results | 2 (14.3) | 18 (35.3) | 47 (90.4) | 17 (70.8) | |
| NDRG4 12m | | | | | 0.489 |
| Positive results | 2 (14.3) | 3 (5.9) | 2 (3.8) | 1 (4.2) | |
| Negative results | 12 (85.7) | 48 (94.1) | 50 (96.2) | 23 (95.8) | |
| NDRG4 34b | | | | | 0.050 |
| Positive results | 2 (14.3) | 3 (5.9) | 1 (1.9) | 4 (16.7) | |
| Negative results | 12 (85.7) | 48 (94.1) | 51 (98.1) | 20 (83.3) | |
| FOBT | | | | | 0.047 |
| Positive results | 6 (42.9)[a] | 9 (17.6)[a,b] | 5 (9.6)[b] | 4 (16.7)[a,b] | |
| Negative results | 8 (57.1) | 42 (82.4) | 47 (90.4) | 20 (83.3) | |

AP: adenomatous polyp; HGIN: high-grade intraepithelial neoplasia. LGIN: low-grade intraepithelial neoplasia; HP: hyperplastic polyp. The same marker letters indicate no statistical difference between the two groups.

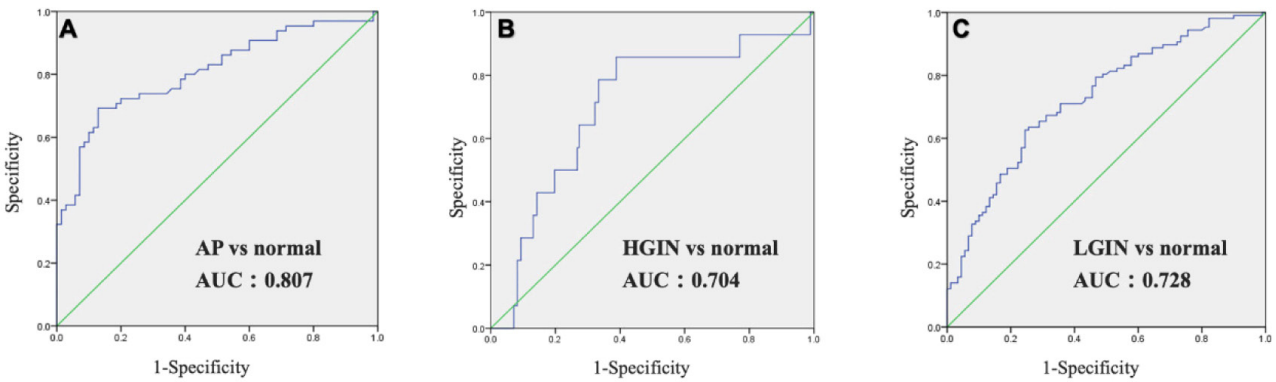


**Figure 2.** Diagnostic performance of NDRG4 12b methylation. **A:** ROC curves for NDRG4 12b methylation in detecting AP. **B:** ROC curves for NDRG4 12b methylation in detecting HGIN. **C:** ROC curves for NDRG4 12b methylation in detecting LGIN.

**Table 4.** The relationship between NDRG4 12b positive results and lesion location, classification

| | *NDRG4 12b* | | *p value* |
|---|---|---|---|
| | *Positive results* n (%) | *Negative results* n (%) | |
| Location | | | 0.827 |
| Left colon | 41 (49.4) | 57 (46.7) | |
| Right colon | 24 (28.9) | 34 (27.9) | |
| Full colon | 18 (21.7) | 31 (25.4) | |
| Paris classification | | | 0.000 |
| 0-Ip | 12 (85.7) | 34 (31.8) | |
| 0-Is | 2 (14.3) | 42 (39.3) | |
| 0-IIa | 0 (0) | 31 (29.0) | |
| Yamada classification | | | 0.747 |
| I | 38 (45.8) | 63 (51.6) | |
| II | 26 (31.3) | 33 (27.0) | |
| III | 12 (14.5) | 19 (15.6) | |
| IV | 7 (8.4) | 7 (5.7) | |

12b methylation was 85.7% for advanced AP, 62.6% for non-advanced AP.

CRC incidence is rising in persons under 50 years, and thorough diagnostic evaluation of young persons with suspected colorectal bleeding is recommended [17,18]. The new Colorectal Cancer Screening Guidelines issued by the American Cancer Society in 2018 updated the age at initiation CRC screening from 50 years to 45 years [19]. In recent years, people has paid more and more attention to polyps, and adenomatous polyps are considered as a kind of precancerous lesions. If polyps are found, endoscopic biopsy should be performed and the polyps should be removed as soon as possible. At present, endoscopy is still the gold standard for diagnosing the presence of intestinal lesions. However, most people cannot tolerate the pain of bowel cleanings and colonoscopy, and some people may miss the best time to detect polyps, thus delaying their illness. Chinese CRC Screening Guidelines [20] recommend FOBT as the primary screening method for average-risk adults 50-75 years old. Although FOBT is widely used for CRC screening, it has limitations in detecting early-stage CRC and advanced adenoma. Recently, DNA methylation testing has emerged as a new molecular approach. The compliance and performance of DNA methylation testing were superior to FOBT [21]. Most studies at home and abroad have shown that genetic testing has high sensitivity and specificity for CRC screening, but modern medical treatment is more inclined to early detection, resection of lesions in the precancerous stage of CRC. Previous studies have shown that NDRG4 is a candidate methylation marker for adenomas screening, the sensitivity and specificity of which were higher than 70% [22], and our reaults are close to this figure. Researchers have demonstrate that combination of fecal DNA testing with FOBT may provide an alternative screening strategy for colorectal neoplasia [23,24], which may also increase the detection rate of precancerous lesions. The patients in the study all had a stool DNA testing before their colonoscopy. In January of this year, we conducted stool DNA testing on 36 patients with high-grade dysplasia 2 to 18 months after polypectomy in our hospital and found that only 4 of them showed positive results for NDRG4 12b methylation 3 months, 12 months, 16 months and 18 months respectively after polypectomy. As the patients haven't the re-examination by colonoscopy, it is not clear whether intestinal polyps exist. This suggests that DNA testing can be used not only to screen for colorectal lesions, but also as a means of review after polypectomy.

## Conclusions

In conclusion, highly sensitive fecal DNA testing of NDRG4 12b methylation is a promising marker for detection of colorectal precancerosis, especially in detecting adenoma. However, as our sample size was not large enough and the number of cases in pathological groups was not uniform, the current results still need to be verified by a large number of further studies.

## Acknowledgements

The present study was supported by the National Natural Science Foundation of China

(No.82000529), Natural Science Foundation of Jiangsu Province (No.SBK2020042595), National Natural Science Foundation of China (No. 81970499), National Science and Technology Major Project (AD18), Jiangsu medical leading talent and innovation team (CXTDA2017033), Provincial science and technology department clinical frontier technology (BE2020784), and Science and Technology Development Fund of Shanghai Pudong New Area (PKX2019-S18).

## Conflict of interests

The authors declare no conflict of interests.

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