NEUROG1 methylation detection in stool DNA: a potential screening biomarker for colorectal cancer

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Running Title: NEUROG1 methylation in stool DNA

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Abstract

Background: To assess the performance of NEUROG1 methylation in the colorectal cancer auxiliary diagnosis.

Methods: The NEUROG1 methylation in tissue and stool samples from patients with colorectal cancer (CRC), advanced adenoma (AA), and non-advanced adenoma (NAA) were evaluated using methylation-specific quantitative PCR.

Results: In tissue samples, the NEUROG1 methylation detection rates were 36% for CRC, 24% for NAA, and 88% for AA. In stool samples, the NEUROG1 methylation detection had a sensitivity of 62.86% for CRC with a PPV of 86.72%. The overall diagnostic specificity of the test for the NAA and the healthy control was 77.92%, with an NPV of 41.10%.

Conclusion: NEUROG1 methylation detection can be used in the CRC and AA auxiliary diagnosis.

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Keywords: Colorectal cancer, NEUROG1, stool, DNA methylation, diagnosis

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide. According to the latest statistics, there are approximately 104,270 new cases of CRC in the United States annually, making it the third most common form of cancer in that country. In addition, the mortality rate is the second-highest among all cancer-related deaths in the United States (Siegel and Miller, 2021). In China, the incidence of CRC ranks fourth after lung cancer, breast cancer, and stomach cancer, and mortality is also fourth among all cancers (Du et al., 2017). Furthermore, the prognosis is poor for patients with advanced metastatic CRC, which has a five-year survival rate of less than 10%. However, most patients with CRC can benefit from surgery when diagnosed early, and the five-year survival rate of TNM stage 1 CRC is greater than 90% (Werner et al., 2016). Therefore, the early screening and diagnosis of CRC play a critical role in positive clinical outcomes. Recently, several countries have initiated CRC screening programs, and a series of consensuses were published on the early screening guidelines of CRC (2021).

The most common methods for CRC screening include colonoscopy, fecal occult blood testing (FOBT), and fecal immunochemical test (FIT).

FOBT comprises hydrogen peroxide for detecting fecal occult blood, known as the guaiac-based FOBt (gFOBt). The fecal immunochemical test (FIT) is based on immunochemistry, FIT has had a distinct advantage over gFOBt: its lack of dietary restrictions prior to sample collection (Benton et al., 2015). FIT has gradually replaced gFOBt in the clinic, but studies have indicated its effectiveness is relatively limited to early-stage CRC diagnosis (Tepus and Yau, 2020). Hence, there is an urgent need for newer screening systems for early-stage CRC.

Colonoscopy is the "gold standard" diagnostic method for CRC due to its high sensitivity and specificity; however, it is invasive, requires skilled technical expertise, and patient noncompliance (Ziegler et al., 2010).

Accumulated evidence has indicated that CRC is a genetic-driven disease driven by DNA mutations, chromatin abnormalities, and epigenetic changes that influence the expression of critical oncogenes (Dickinson et al., 2015; Huang et al., 2018). Epigenetic changes, such as abnormal DNA methylation, non-coding RNA (miRNA and siRNA), and histone modifications, are closely associated with CRC development and progression (Okugawa et al., 2015).

Abnormal DNA hypomethylation in the promoter regions of tumor suppressor genes was reported to be an early event in CRC. For example, DNA hypomethylation of the promoter region of Secreted frizzled-related protein 2 *SFRP2* gene activates the Wnt signalling pathway and promotes tumorigenesis in CRC (Zhang et al., 2014).

Studies have shown that genetic methylation biomarkers can be detected in body fluids, such as blood, urine, and stool. Hence, they may serve as novel biomarkers for CRC screening in the future. Furthermore, stool samples from patients with cancer often contain more DNA than blood, as tumor cells can be shed off from mass and excreted through the stool. Studies have shown that the sensitivity of stool samples is significantly higher than plasma samples (Ahlquist et al., 2012a).

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In the last decade, stool DNA methylation detection has become a non-invasive, highly specific, and cheap screening method for diagnosing CRC. In 2014, the United States Food and Drug Administration (FDA) approved a multi-target stool DNA (MT-sDNA) test for screening CRC among high-risk asymptomatic patients (Imperiale et al., 2014).

Recently, it was demonstrated that abnormal methylation of genes adenomatous polyposis coli (*APC*), ataxia telangiectasia mutated (*ATM*), cyclin-dependent kinase inhibitor 2A(*CDKN2A*), GATA binding protein 4 (*GATA4*), and secreted frizzled-related protein 2 (*SFRP2*) could be used for CRC screening in stool samples (Kadiyska and Nossikoff, 2015; Laugsand et al., 2021).

Neurogenin 1 (NEUROG1) is one of the classic methylation biomarkers that can distinguish the degree of the CpG island methylator phenotype (CIMP) (Ibrahim et al., 2011). Previous studies have shown that the methylation of NEUROG1 could be used as a serum biomarker for early-stage CRC (Herbst et al., 2011; Otero-Estévez and Gallardo-Gomez, 2020). However, it has not been investigated for stool samples.

This study aimed to evaluate the performance of the NEUROG1 methylation test in screening CRC. Herein, we detected NEUROG1 methylation levels in stool samples and tumor specimens from CRC patients.

1 Materials and Method

1.1 sample selection

In this study, The formalin-fixed, paraffin-embedded (FFPE) samples were collected and stored at room temperature from a total of 75 patients, 25 with CRC, 25 with non-advanced adenomas (NAA), and 25 with advanced adenomas (AA) were diagnosed at the First Affiliated Hospital of Henan University of Science and Technology from May 2019 to May 2020 the patients were confirmed by two experienced doctors, depending on the colonoscopy results and along with the pathology results of CRC, AA, and NAA patients,

Stool samples were collected from 274 patients diagnosed and treated at Fujian Provincial Hospital from July 2019 to December 2023, including 105 CRC patients, 92 AA patients, and 39 NAA patients.

None of the patients had received any anticancer treatment before admission. Control stool samples were collected from 38 healthy individuals undergoing colonoscopy, and CRC, AA, NAA patients were excluded.

In addition, several clinical characteristics were collected, including age, sex, and classification.

This study was approved by the Institutional Review Board of Fujian Provincial Hospital (K2019-11-027). Informed consent was obtained from all enrolled patients and healthy control subjects.

Stool samples were collected before tumor resection and stored at -80 °C in a storage buffer.

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1.2 DNA extraction from stool

Stool DNA was extracted according to the operation manual and published protocol using a DNA extraction kit in the stool (Ahlquist et al., 2012b). After thawing, the buffered stool samples were homogenized with an oscillator and centrifuged. Then, an aliquot of 12 mL stool supernatant was treated with 50 mg/mL polyvinyl polypyrrolidone PVPP (Aladdin, Shanghai, China).

The target gene sequence was directly captured by hybridization with the oligonucleotide probe (CGTGCAGCGCCCGGGTATTTGCATAATTTATGCTCGCGGGAGGCCGCCATCGCCCCTCCCCCAACCCGGAGTGTGCCCGTAATTACCG).

For this purpose, 10 mL polyvinylpolypyrrolidone-treated supernatant was denatured using 2.4M (molar) guanidine isothiocyanate at 92°C for 15 min (Aladdin, Shanghai, China). Next, 50 µL oligonucleotide capture probe-modified carboxyl magnetic beads (JSR) were added, mixed, and incubated at room temperature for 30 minutes.

The beads were collected using the magnetic rack and washed with washing buffer (10 mM MOPS,150 mM NaCl, pH 7.5) three times. Finally, 50 µL nuclease-free water containing 20 ng/µL transfer RNA (Merck K GaA, Darmstadt, Germany) was added to the eluted DNA.

The 50 µL elution was used for bisulfite conversion, and the purified DNA was eluted to 60 µL in the elution buffer. The bisulfite transformation Kit (Zymo Research, Irvine, CA, USA) was used for DNA bisulfite transformation and purification of transformation products. All steps were performed following the manufacturer's instructions.

1.3 DNA extraction of FFPE specimens

TIANamp FFPE DNA Kit (Tiangen Biotech Co., Ltd, Beijing, China) was used to isolate DNA from FFPE tissue samples. Briefly, 4-5 sections of FFPE specimens were collected in 1.5mL centrifuge tubes. Deparaffinization and dehydration were performed by adding dimethylbenzene and 100% ethanol. After air drying, 400 µL digest buffer with 20 µL proteinase K was added to the precipitation. Then, the precipitation was suspended and digested at 55 °C for one hour. Following a one-hour incubation at 90°C, the suspension was added into spin columns. After cleaning and centrifugation, the spin columns were dried and eluted with 50-100 µL elution buffer. The DNA samples were stored at -20 °C.

After extraction, the concentration of DNA was determined with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc., California, USA).

1 µg of extracted DNA was treated with bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA).

1.4 Methylation-specific Quantitative PCR (MSP)

The methylation status of NEUROG1 was evaluated by MSP (Suzhou MicroDiag Biomedicine Co., Ltd, Suzhou, China). Sequences of the primers and probes for NEUROG1 and β-Actin (*ACTB*) were designed and synthesized by GENEWIZ

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Stool DNA was extracted according to the operation manual and published protocol using a DNA extraction kit in the stool (Ahlquist et al., 2012b). Briefly, immediately a

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(Suzhou, China). The *ACTB* gene is located on **chromosome 7 (7p22.1)**, and NEUROG1 on chromosome 5 (5q31.1) was used as a reference. The sequences of the primers and probes for the indicated genes are as follows: NEUROG1_Forward: TCGTGTAGCGTTCGGGT, NEUROG1_Reverse: CACTCCGAATTAAAAAAAAAACG, NEUROG1_probe: ATCCCGCGAACATA; ACTB_Forward: GTGATGGAGGAGGTTTAGTAAGTT, ACTB_Reverse: CCAATAAAACCTACTCCTCCCTTAA, ACTB_probe: ACCACCACCCAACACACAATAACAAACACA.

In brief, the total volume of qPCR was 30 µL, including 15 µL of DNA sample and 15 µL of PCR master mix. Next, qPCR was conducted on a LightCycler480 II thermal cycler (Roche Diagnostics Corporation, Indianapolis, USA) using the following conditions: denaturation 95 ºC for 20 min on cycle; 45 cycles (annealing 95 ºC for 20s and extension 60 ºC for 35 s; and finally chilled to 40 ºC for 30 s).

After the amplification, the data were analyzed on LC480-II (Roche Diagnostics Corporation, Indianapolis, USA).

A standard curve elaborated for NEUROG1 methylation to calibrate the investigation system (100%-0% bisulfite-converted NEUROG1-positive genomic DNA; slope = -0.2762; $R^2 = 0.9906$). In brief, bisulfite conversion was performed of genomic DNA extracted from cells, in which the NEUROG1 promoter region was fully methylated (HCT116 cells) or unmethylated (293T cells) using a commercially available kit (EZ DNA Methylation-Gold Kit, Enzo, USA). Then, bisulfite-converted fully methylated genomic DNA was mixed with bisulfite-converted unmethylated DNA to obtain a bisDNA gradient (where NEUROG1 methylated DNA was 100%, 50%, 25%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, and 0% to the total mixture), while the whole mixture's bisDNA concentration was maintained at two ng/µL. After that, the samples were subjected to amplification, and the results were plotted to generate the standard curve. Negative and positive cell DNA were purchased from Fubio Biotechnology, Co., Ltd (Suzhou, China).

The quality control's cycle threshold/crossing point (CP) value is 27-30 for tissue methylation DNA. For stool DNA detection, the CP value of the quality control needs to be <36. For samples without amplification in the target channels, 45 (maximum amplification cycle number of PCR) should be taken for the fitting calculation.

SPSS software (IBM Corp., Armonk, NY, USA) was used to conduct the fitting logistic regression analysis of CP values for target genes and internal reference genes, and the fitting formula was obtained:

$$\frac{1}{1+e^{-(8.135-0.101*\mathrm{NEUROG1}-0.098*\mathrm{ACTB})}}$$

The sample is positive if the fitting value is >0.7848.

1.5 Statistical analysis

Statistical analysis was performed using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). Receiver operating characteristic (ROC) curves, constructed by the pROC package (Kuang et al., 2020), were employed to determine the marker's discriminative capacity, providing the area under the curve (AUC). The cut-off selected

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resulted from setting the confidence interval close to 95%. The detection performance of stool NEUROG1 gene methylation in CRC and precancerous lesions was investigated. All the data were presented in percentages (%). The Chi-square test was used to compare the data, and *P*-values <0.05 were considered statistically significant.

2 Results

2.1 NEUROG1 methylation detection in FFPE specimens

To elucidate the methylation status of the promoter region of NEUROG1 in colon cancer, MSP was conducted in both FFPE specimens and patients' feces samples. We determined the detection rates of NEUROG1 methylation in 75 FFPE samples, including (25 CRC, 25 AA, and 25 NAA) as shown in (Table 1). The methylation rate for CRC was 36%, and for NAA was 24%. Notably, the methylation level of AA was 88% and exhibited positive NEUROG1 methylation. Moreover, the CP values were in acceptable ranges for all samples. Hence, these findings revealed that NEUROG1 has the potential to be a biomarker for precancerous colon lesions.

2.2 Stool DNA-NEUROG1 methylation detection in CRC

The methylation level of the NEUROG1gene in stool samples was evaluated by collecting stool samples from 274 patients, including (105 CRC, 92 AA, 39 NAA) and 38 healthy cohorts. The clinical characteristics of these patients are listed in **Table 2**. The ages ranged from 24 to 87, with 154 males and 115 females. Next, NEUROG1 methylation in stool samples was tested in different cancer stages.

In the 105 CRC patients, the NEUROG1 methylation level was 62.86%. To further evaluate the sensitivity of NEUROG1 methylation for different tumor classifications and positions, the methylation level was assessed in various CRC stages (I, II, III, IV), as shown in (**Table 3**).

In the 95 patients with known stages, the detection rate ranged from 41.18% to 70.37%, with no significant differences between each subgroup. The methylation rates of different tumor positions were explored among 101 patients. The methylation rate of proximal colorectal cancer was 46.42%, and for distal colorectal cancer, it was 67.12%. However, the methylation rates in CRC patients with distal CRC seemed higher, and the difference was not statistically significant, as shown in (**Table 3**).

2.3 Stool DNA-NEUROG1 methylation detection in AA and NAA

In 92 cases of AA, the methylation level was 48.91%. Further analysis was conducted to compare the rates in different subtypes of AA based on the tumor position and size. *NEUROG1* methylation rates of proximal and distal AA were 38.71% and 57.41%, respectively (*P*>0.05).

Additionally, when the diameter of the adenoma was ≥3 cm, the detection rate increased significantly, reaching up to 66.67%, as shown in (**Table 4**). However, there were no significant differences between the different tumor sizes.

In the 39 NAA cases, 26 samples were negative, resulting in a detection specificity of 66.67%.

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2.4 stool DNA methylation Level in healthy control

In the 38 healthy volunteers, 34 samples were negative, resulting in a detection specificity of 89.47%. Hence, the NEUROG1 methylation detection sensitivity was 56.35% (111/197) in the positive group of 197 CRC and AA. By comparison, the detection specificity was 77.92% (60/77) in the cases of NAA and healthy people in the control group.

2.4 Diagnostic performance of NEUROG1 methylation detection with stool samples

Receiver operating characteristic (ROC) curves were elaborated to evaluate the discriminatory capacity of NEUROG1, as shown in (**Figure 2**). In our study, 274 stool samples were subjected to NEUROG1 methylation detection. Among those from NAA, healthy individuals were selected as controls, and ROC curve analyses were performed. As shown in **Figure 2**, the AUC for the NEUROG1 detection of CRC and AA was 0.718 (95% CI 0.652-0.781).

In conclusion, in the positive group of CRC and AA, the sensitivity value of NEUROG1 methylation was 56.35%， PPV 86.72%， PLR2.55. In the control group of NAA and healthy volunteers, the specificity value of NEUROG1 methylation detection was 77.92%， NPV41.10%， NLR0.56, as shown in(**Table 5**).

3 Discussion

Methylation in the promoter region of *NEUROG1* was identified long ago, and applications of NEUROG1 methylation in blood DNA tests have been reported in CRC (Goel et al., 2010; Herbst et al., 2011; Li et al., 2018). However, studies of NEUROG1 methylation in the stool have not been reported. This study was the first to systematically study the methylation of NEUROG1 in stool and tissue samples from patients with CRC, AA, and NAA.

CRC development is a complex process of five steps, including normal intestinal epithelium, NAA, AA, adenocarcinoma, and finally, cancer metastasis (Siraj et al., 2014). This process is associated with a large number of oncogene and tumor suppressor gene disturbances, including mutations of APC, Kirsten rat sarcoma viral oncogene homolog (KRAS), and Tumor Protein P53 (TP53), microsatellite instability, and DNA methylation abnormalities (Okugawa et al., 2015).

In patients with CRC, a proportion of tumor cells can be shed from the gut into the stool (Diehl et al., 2008). Therefore, the preliminary for CRC can be conducted by detecting the DNA shedding in stool samples. Previously, Sidransky and colleagues discovered the presence of the KRAS gene in stool samples from CRC patients in 1992 (Sidransky et al., 1992). Unfortunately, the sensitivity of the DNA mutation detection in stool samples was too low, and screening was difficult

Many studies have reported the role of DNA methylation and suggested that DNA methylation detection could be a great approach for screening early-stage CRC (Barault et al., 2018). However, in a previous report, the sensitivity of DNA methylation detection for a single gene in the early stages of the disease was low (Zhao et al., 2020).

Various DNA methylation molecular targets could be used as biomarkers in CRC.

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For example, one study explored the role of Syndecan-2 (SDC2) methylation in CRC and found that SDC2 methylation could be detected in 81.1% (159/196) CRC and 58.2% (71/122) adenomas in stool samples (Niu et al., 2017). In addition, the tumor suppressor gene, tissue factor pathway inhibitor 2 (TFPI2), was reported to be methylated in CRC. The sensitivity of methylated TFPI2 in stool DNA of stage I-III CRC patients was 76% to 89%, with a specificity of 79% to 93% (Glöckner et al., 2009). Recently, one report implied that potassium voltage-gated Channel Subfamily Q Member 5 (KCNQ5) methylation and Chromosome 9 Open Reading Frame 50 (C9orf50) methylation in stool DNA could be possible biomarkers for CRC detection, with sensitivities of 77.3% and 85.9%, and specificities of 91.5% and 95.0%, respectively (Niu et al., 2017). In addition, one study explored the application of Phosphatase and Actin Regulator 3 (PHACTR3) methylation in CRC stool DNA, with a sensitivity of 55% (95% CI: 33-75) and specificity of 95%. In our study, we investigated the role of methylated NEUROG1 in the stool DNA of CRC patients and found the sensitivity was 62.86% and specificity was 77.92%. In addition, accumulating evidence has evaluated stool-based DNA methylation markers for CRC diagnosis. Although methylated genes, such as SDC2, SFRP2, and TFPI2, have been demonstrated to possess the capacity to detection of established cancers (Glöckner et al., 2009; Zhang et al., 2014; Sun et al., 2019; Wang et al., 2020), the goal of effective diagnosis has not been achieved yet. For instance, the most known methylation marker approved by the FDA is SEPT9. A prospective analysis of SEPT9 methylation detection showed a sensitivity of 48.2% for CRC and a sensitivity of only 11.2% for AA (Church et al., 2014). These findings have compelled us to consider evaluating multiple markers for a more precise diagnosis.

In this study, the detection rate of NEUROG1 methylation in AA FFPE tissue was significantly higher than in CRC and NAA tissue. We speculate that NEUROG1gene methylation may occur in precancer or early-stage cancer and participate in the process of tumor progression.

The overall NEUROG1 methylation detection sensitivity for CRC in stool samples was 62.86%. The detection rate of stage I-III was similar, whereas the detection rate of stage IV was relatively low, which may be due to the small number of cases (17 cases). It is also possible that the gene becomes unmethylated due to several factors in stage IV. The sample size should be increased in further study.

In stool samples, the overall NEUROG1 methylation detection sensitivity for AA was 48.91%. The detection rate of AA size was comparable in the≤1.9cm and 2-2.9cm groups. In the AA size≥3cm, the hypermethylation was significant.

Currently, the research on *NEUROG1* methylation in CRC mainly focuses on serum to detect the early stage of CRC (Herbst et al., 2011). Studies of NEUROG1 methylation in the stool have not been reported. Our study initially clarified its detection performance and laid a certain foundation for the later development of detection methods using this gene or the combination of this gene with the other genes. This study also has some limitations. First, future studies should evaluate the diagnostic performance of stool DNA testing in large cohorts of NAA patients, healthy volunteers and patients with other upper gastrointestinal diseases, inflammatory bowel diseases and liver cancer patients. Second, most of the samples are from Fujian Provincial

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Hospital, which may limit the representativeness of the findings. Further studies with large sample sizes are needed to provide more reliable evidence for the clinical application of combined methylation detection.

4 Conclusions

In conclusion, NEUROG1 methylation has a good sensitivity in detecting CRC and precancer, especially in detecting the precancerous stage. Therefore, NEUROG1 has the potential to serve as a marker alone or in combination with other markers for CRC auxiliary diagnosis.

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Competing interests

Tao Wang, Yan Lu, Zhaofen Ba, Xiaowei Cheng and Tiantian Zhang are employed by Jiangsu Microdiag Biomedical Technology Co., LTD. All other authors declare no competing interests.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Fujian Provincial Hospital (K2019-11-027). Informed consent was obtained from all enrolled patients and healthy control subjects.

Availability of data and materials

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

Deleted: analysed

Authors' contributions

Conceptualization: MHC, and TW; Validation/ Investigation/ Formal analysis/ Writing-original draft, editing: LZ, AL, JC, XC, ZB, and YL; Resource: JL, MSC, YW, TZ and XY. All authors have read and agreed to the published version of the manuscript.

REFERENCES

Ahlquist DA, Taylor WR, Mahoney DW, Zou H, Domanico M, Thibodeau SN, Boardman LA, Berger BM, and Lidgard GP. 2012a. The stool DNA test is more accurate than the plasma septin 9 test in detecting colorectal neoplasia. *Clin Gastroenterol Hepatol* 10: 272-277.e271. doi: 10.1016/j.cgh.2011.10.008.

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, Sørensen HT, Berger BM, and Lidgard GP. 2012b. Next-generation stool DNA test accurately detects colorectal cancer and large adenomas. *Gastroenterology* 142: 248-256; quiz e225-246. doi: 10.1053/j.gastro.2011.10.031.

Barault L, Amatu A, Siravegna G, Ponzetti A, Moran S, Cassingena A, Mussolin B, Falcomatà C, Binder AM, Cristiano C, Oddo D, Guarrera S, Cancelliere C, Bustreo S, Bencardino K, Maden S, Vanzati A, and Zavattari P. 2018. Discovery of methylated circulating DNA biomarkers for comprehensive non-invasive monitoring of treatment response in metastatic colorectal cancer. *Gut* 67: 1995-2005. doi: 10.1136/gutjnl-2016-313372.

Benton SC, Seaman HE, and Halloran SP. 2015. Faecal occult blood testing for colorectal cancer screening: the past or the future. *Curr Gastroenterol Rep* 17: 428. doi: 10.1007/s11894-015-0428-2.

Church TR, Wandell M, Lofton-Day C, Mongin SJ, Burger M, Payne SR, Castanos-Velez E, Blumenstein BA, Rosch T, Osborn N, Snover D, Day RW, Ransohoff

DF, Presept Clinical Study Steering Committee I, and Study T. 2014. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut* 63: 317-325. doi: 10.1136/gutjnl-2012-304149.

Dickinson BT, Kisiel J, Ahlquist DA, and Grady WM. 2015. Molecular markers for colorectal cancer screening. *Gut* 64: 1485-1494. doi: 10.1136/gutjnl-2014-308075.

Diehl F, Schmidt K, Durkee KH, Moore KJ, Goodman SN, Shuber AP, Kinzler KW, and Vogelstein B. 2008. Analysis of mutations in DNA isolated from plasma and stool of colorectal cancer patients. *Gastroenterology* 135: 489-498. doi: 10.1053/j.gastro.2008.05.039.

Du LB, Li HZ, Wang YQ, Zhu C, Zheng RS, Zhang SW, Chen WQ, and He J. 2017. [Report of colorectal cancer incidence and mortality in China, 2013]. *Zhonghua Zhong Liu Za Zhi* 39: 701-706. doi: 10.3760/cma.j.issn.0253-3766.2017.09.012.

Glöckner SC, Dhir M, Yi JM, McGarvey KE, Van Neste L, Louwagie J, Chan TA, Kleeberger W, de Bruïne AP, Smits KM, Khalid-de Bakker CA, Jonkers DM, Stockbrügger RW, Meijer GA, Oort FA, Iacobuzio-Donahue C, Bierau K, Herman JG, Baylin SB, Van Engeland M, Schuebel KE, and Ahuja N. 2009. Methylation of TFPI2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer. *Cancer Res* 69: 4691-4699. doi: 10.1158/0008-5472.can-08-0142.

Goel A, Xicola RM, Nguyen TP, Doyle BJ, Sohn VR, Bandipalliam P, Rozek LS, Reyes J, Cordero C, Balaguer F, Castells A, Jover R, Andreu M, Syngal S, Boland CR, and Llor X. 2010. Aberrant DNA methylation in hereditary nonpolyposis colorectal cancer without mismatch repair deficiency. *Gastroenterology* 138: 1854-1862. doi: 10.1053/j.gastro.2010.01.035.

Herbst A, Rahmig K, Stieber P, Philipp A, Jung A, Ofner A, Crispin A, Neumann J, Lamerz R, and Kolligs FT. 2011. Methylation of NEUROG1 in serum is a sensitive marker for the detection of early colorectal cancer. *Am J Gastroenterol* 106: 1110-1118. doi: 10.1038/ajg.2011.6.

Huang D, Sun W, Zhou Y, Li P, Chen F, Chen H, Xia D, Xu E, Lai M, Wu Y, and Zhang H. 2018. Mutations of key driver genes in colorectal cancer progression and metastasis. *Cancer Metastasis Rev* 37: 173-187. doi: 10.1007/s10555-017-9726-5.

Ibrahim AE, Arends MJ, Silva AL, Wyllie AH, Greger L, Ito Y, Vowler SL, Huang TH, Tavaré S, Murrell A, and Brenton JD. 2011. Sequential DNA methylation changes are associated with DNMT3B overexpression in colorectal neoplastic progression. *Gut* 60: 499-508. doi: 10.1136/gut.2010.223602.

Imperiale TF, Ransohoff DF, Itzkowitz SH, Levin TR, Lavin P, Lidgard GP, Ahlquist DA, and Berger BM. 2014. Multitarget stool DNA testing for colorectal-cancer screening. *N Engl J Med* 370: 1287-1297. doi: 10.1056/NEJMoa1311194.

Kadiyska T, and Nossikoff A. 2015. Stool DNA methylation assays in colorectal cancer screening. *World J Gastroenterol* 21: 10057-10061. doi:

10.3748/wjg.v21.i35.10057.

Kuang Y, Wang Y, Zhai W, Wang X, Zhang B, Xu M, Guo S, Ke M, Jia B, and Liu H. 2020. Genome-Wide Analysis of Methylation-Driven Genes and Identification of an Eight-Gene Panel for Prognosis Prediction in Breast Cancer. *Front Genet* 11: 301. doi: 10.3389/fgene.2020.00301.

Laugsand EA, Brenne SS, and Skorpen F. 2021. DNA methylation markers detected in blood, stool, urine, and tissue in colorectal cancer: a systematic review of paired samples. *Int J Colorectal Dis* 36: 239-251. doi: 10.1007/s00384-020-03757-x.

Li W, Zhao LZ, Ma DW, Wang DZ, Shi L, Wang HL, Dong M, Zhang SY, Cao L, Zhang WH, Zhang XP, Zhang QH, Yu L, Qin H, Wang XM, and Chen SL. 2018. Predicting the risk for colorectal cancer with personal characteristics and fecal immunochemical test. *Medicine (Baltimore)* 97: e0529. doi: 10.1097/MD.0000000000010529.

Niu F, Wen J, Fu X, Li C, Zhao R, Wu S, Yu H, Liu X, Zhao X, Liu S, Wang X, Wang J, and Zou H. 2017. Stool DNA Test of Methylated Syndecan-2 for the Early Detection of Colorectal Neoplasia. *Cancer Epidemiol Biomarkers Prev* 26: 1411-1419. doi: 10.1158/1055-9965.epi-17-0153.

Okugawa Y, Grady WM, and Goel A. 2015. Epigenetic Alterations in Colorectal Cancer: Emerging Biomarkers. *Gastroenterology* 149: 1204-1225.e1212. doi: 10.1053/j.gastro.2015.07.011.

Otero-Estévez O, and Gallardo-Gomez M. 2020. Value of Serum NEUROG1 Methylation for the Detection of Advanced Adenomas and Colorectal Cancer.

Diagnostics (Basel) 10: 437. doi: 10.3390/diagnostics10070437.

Pankiewicz K, Fijałkowska A, Issat T, and Maciejewski TM. 2021. Insight into the Key Points of Preeclampsia Pathophysiology: Uterine Artery Remodeling and the Role of MicroRNAs. *Int J Mol Sci* 22: 3132. doi: 10.3390/ijms22063132.

Sidransky D, Tokino T, Hamilton SR, Kinzler KW, Levin B, Frost P, and Vogelstein B. 1992. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. *Science* 256: 102-105. doi: 10.1126/science.1566048.

Siegel RL, and Miller KD. 2021. Cancer Statistics. *CA Cancer J Clin* 71: 7-33. doi: 10.3322/caac.21654.

Siraj AK, Bu R, Prabhakaran S, Bavi P, Beg S, Al Hazmi M, Al-Rasheed M, Alobaisi K, Al-Dayel F, AlManea H, Al-Sanea N, Uddin S, and Al-Kuraya KS. 2014. A very low incidence of BRAF mutations in Middle Eastern colorectal carcinoma. *Mol Cancer* 13: 168. doi: 10.1186/1476-4598-13-168.

Sun M, Liu J, Hu H, Guo P, Shan Z, Yang H, Wang J, Xiao W, and Zhou X. 2019. A novel panel of stool-based DNA biomarkers for early screening of colorectal neoplasms in a Chinese population. *J Cancer Res Clin Oncol* 145: 2423-2432. doi: 10.1007/s00432-019-02992-2.

Tepus M, and Yau TO. 2020. Non-Invasive Colorectal Cancer Screening: An Overview. *Gastrointest Tumors* 7: 62-73. doi: 10.1159/000507701.

Wang J, Liu S, Wang H, Zheng L, Zhou C, Li G, Huang R, Wang H, Li C, Fan X, Fu X, Wang X, Guo H, Guan J, Sun Y, Song X, Li Z, Mu D, Sun J, Liu X, Qi Y, Niu F, Chen C, Wu X, Wang X, Song X, and Zou H. 2020. Robust performance of a novel stool DNA test of methylated SDC2 for colorectal cancer detection: a multicenter clinical study. *Clin Epigenetics* 12: 162. doi: 10.1186/s13148-020-00954-x.

Werner S, Krause F, Rolny V, Strobl M, Morgenstern D, Datz C, Chen H, and Brenner H. 2016. Evaluation of a 5-Marker Blood Test for Colorectal Cancer Early Detection in a Colorectal Cancer Screening Setting. *Clin Cancer Res* 22: 1725-1733. doi: 10.1158/1078-0432.ccr-15-1268.

Zhang H, Zhu YQ, Wu YQ, Zhang P, and Qi J. 2014. Detection of promoter hypermethylation of Wnt antagonist genes in fecal samples for diagnosis of early colorectal cancer. *World J Gastroenterol* 20: 6329-6335. doi: 10.3748/wjg.v20.i20.6329.

Zhao G, Ma Y, Li H, Li S, Zhu Y, Liu X, Xiong S, Liu Y, Miao J, Fei S, Zheng M, and Zhao X. 2020. A novel plasma based early colorectal cancer screening assay base on methylated SDC2 and SFRP2. *Clin Chim Acta* 503: 84-89. doi: 10.1016/j.cca.2020.01.010.

Ziegler M, Schubring-Giese B, Bühner M, and Kolligs FT. 2010. Attitude to secondary prevention and concerns about colonoscopy are independent predictors of acceptance of screening colonoscopy. *Digestion* 81: 120-126. doi: 10.1159/000223448.

Figure Legends

Figure 1: Experimental design in this study. Two separated cohorts were included: the FFPE cohort (N=75) and the stool cohort (N=274). NEUROG1 methylation detection and analysis were performed on all the samples enrolled.

Figure 2: Result of ROC analysis evaluating 274 stool samples undergone NEUROG1 methylation.