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ORIGINAL ARTICLE – CANCER RESEARCH

A novel panel of stool-based DNA biomarkers for early screening of colorectal neoplasms in a Chinese population

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Abstract

Purpose The mortality of colorectal cancer ranked fifth in China according to cancer statistics in 2015. Cancer screening had been repeatedly proved to play a vital role in decreasing the incidence and mortality of colorectal cancer, but the existing screening methods could not meet the requirements. So it is of urgent need to develop a non-invasive, convenient and accurate screening method.

Methods In this study, stool samples were collected from 102 colorectal cancer, 20 colorectal adenoma, 6 hyperplastic polyps patients and 105 normal controls, and stool DNA was extracted for detection of methylation (BMP3, NDRG4, SDC2 and SFRP2) and KRAS mutations. Meanwhile, hemoglobin in stool samples was detected by immunoassays. Then, the logistic regression model used for classification was built with these biomarkers, and a ROC curve was drawn to evaluate the performance of each biomarker and the panel of them. Meanwhile, conventional serum biomarkers were detected for the comparison of positive rate in colorectal cancer between serum biomarkers and stool DNA biomarkers.

Results As a result, a classification model built with methylation of SDC2 and SFRP2, KRAS mutations and hemoglobin showed a sensitivity of 91.4% for colorectal cancer and 60% for adenoma with the specificity of 86.1%. Compared with it, most of the conventional serum biomarkers showed a sensitivity of less than 20% for colorectal cancer which was significantly lower than stool DNA biomarkers.

Conclusions A novel panel comprised of stool DNA biomarkers was of much higher sensitivity and specificity in early screening of colorectal neoplasms than conventional serum biomarkers.

Keywords Stool based · DNA methylation · Early screening · Colorectal neoplasms · Non-invasive

Introduction

In China, the incidence and mortality of cancer have been increasing, making it the leading cause of death since 2010. It has been estimated that the number of colorectal cancer cases and deaths (thousands) were 376.3 and 191.0 in China, 2015, which ranked fifth among all kinds of cancers (Chen et al. 2016). In general, its incidence is low in people younger than 50 years old and increases significantly in people more than 50. But more and more evidences suggested that there was an increasing trend in early-onset colorectal cancer (Siegel et al. 2019; Young et al. 2015). Mostly, colorectal tumorigenesis involves a multistep process including a series of histological, morphological, and genetic changes. Thus, it is feasible to screen precancerous lesions in individuals at average risk for colorectal cancer (CRC) (Simon 2016). There were several screening options for CRC, but each of them had disadvantages and limitations. For example, colonoscopy allowed visualization of full colon and detection of distal and proximal lesions with high sensitivity, and could remove lesions at the same time of detection, but it was invasive and accompanied by unpleasant bowel preparation as well as risks of bowel perforation and bleeding

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(Simon 2016). So, it is difficult to promote the application of colonoscopy among high-risk groups on a large scale. Flexible sigmoidoscopy could really reduce the incidence and mortality of colorectal cancer (Atkin et al. 2010; Shroff et al. 2014), but it could only detect local pathological changes in rectum and left colon. Serum biomarkers, such as CEA and CA199, were always pan cancer biomarkers in the diagnosis of breast cancer (Zheng and Luo 2005), epithelial ovarian cancer (Guo et al. 2017), pancreatic cancer (Ona et al. 1973), colorectal cancer (Wu et al. 1995), and showed low sensitivity in colorectal cancer detection. So, it was urgent to develop a non-invasive, convenient and accurate screening method.

In recent years, studies have indicated that a common event in the tumorigenesis of CRC was global hypomethylation and discrete hypermethylation at the promoter regions of specific genes which were involved in cancer-related processes, such as cell cycle regulation, DNA repair, apoptosis, angiogenesis, adhesion and invasion (Silva et al. 2013). And it suggested the potential use of DNA methylation in detection of colorectal cancer. SEPT9 methylation detection was the first FDA-approved blood-based test for colorectal cancer screening (Devos et al. 2009). As reported, SEPT9 methylated DNA test showed a sensitivity of 90% for screening of colorectal cancer with a high specificity of 88%. And for early stage cancer (I and II), the sensitivity was as high as 87% (Warren et al. 2011). But one prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer showed that methylated SEPT9 detection in plasma showed a sensitivity of 48.2% for colorectal cancer with the specificity of 91.5% and the sensitivity for advanced adenomas was as low as 11.2% (Church et al. 2014). This prospective evaluation suggested that a single biomarker might be insufficient for a high level of sensitivity in different races.

Other than plasma or serum samples, stool samples could also be used for the screening of colorectal cancer and precancerous lesions. Stool testing had a few advantages over other screening methods, including no unpleasant cathartic preparation, no risks of bowel perforation and bleeding, and it could detect precancerous lesions along the full length of colorectum (Osborn and Ahlquist 2005). In addition, stool-based DNA was relatively stable during fecal transport and storage, and it originated from the neoplasm itself, rather than the circulation, which could improve its specificity (Goel 2010). David A. Ahlquist carried out a study to detect colorectal cancer and adenomas by a stool-based DNA test. The stool-based DNA test was implemented by detection of a couple of biomarkers, including methylation of four genes (vimentin, NDRG4, BMP3, TFPI2), KRAS mutations and reference gene ACTB, as well as hemoglobin. Finally, the stool-based DNA test showed a sensitivity of 85% for colorectal cancer patients and 54% for adenomas (≥ 1 cm) patients with specificity of 90% (Ahlquist et al. 2012). Subsequently, Thomas F. Imperiale carried out another non-invasive, multi-target stool DNA test (methylation of BMP3 and NDRG4, KRAS mutations, hemoglobin) and fecal immunochemical test in asymptomatic persons at average risk for colorectal cancer (Imperiale et al. 2014). Of 9989 participants, colorectal cancer amounted to 65 and advanced precancerous lesions accounted to 757. To be specific, multi-target stool DNA test showed a sensitivity of 92.3% for colorectal cancer and 42.4% for advanced precancerous lesions with a specificity of 86.6% (Imperiale et al. 2014). It was much better than FIT in the screening of colorectal cancer and advanced precancerous lesions. However, no more repeated research was implemented in a Chinese population.

Other than the methylated genes mentioned above, there were more methylated genes involved in colorectal cancer screening, such as SFRP2 and SDC2. As reported, DNA methylation of SFRPs could be detected in colorectal cancer and adenomas tissue, but not in normal colorectal mucosa tissue (Oberwalder et al. 2008). And SFRP2 methylation in fecal DNA increased significantly from healthy controls to patients with hyperplastic polyps and adenomas (Oberwalder et al. 2008). In a systematic meta-analysis, the pooled sensitivity, specificity and AUC (area under the curve) of methylated SFRP2 in stool of patients with colorectal cancer vs. healthy subjects were 0.71, 0.94 and 0.94, respectively (Yang et al. 2016). It suggested that SFRP2 methylation in stool was a potential non-invasive biomarker for colorectal cancer screening. Besides, stool-based SDC2 methylation test was also a potential non-invasive diagnostic tool for early screening of colorectal cancer (Niu et al. 2017) and the overall sensitivity was 90.0% and 33.3% for detecting colorectal cancer and small polyps, respectively, with a specificity of 90.9% (Oh et al. 2017). Similarly, no more repeated research of methylation of SDC2 and SFRP2 for screening of colorectal cancer was implemented in a Chinese population.

To verify the applications of these novel potential stool-based biomarkers in colorectal neoplasms screening and diagnosis, especially in a Chinese population, a total of 233 stool samples from colorectal cancer (105), colorectal adenoma (20), hyperplastic polyps (6) and normal controls (102) were collected, and the level of a few biomarkers, including KRAS mutations (Battaglia et al. 2014; Roa et al. 2013), methylation of BMP3, NDRG4, SDC2, SFRP2 and hemoglobin were detected and used to build a classification model for colorectal neoplasms screening. Finally, a panel of stool-based biomarkers was coupled to be a screening method of colorectal cancer and adenoma patients with higher sensitivity and specificity, which was expected to reduce the incidence and mortality of colorectal cancer in a Chinese population.

Materials and methods

Patients and samples

This study was approved by the Institutional Review Board at the first affiliated hospital of Soochow university. A total of 105 colorectal cancer patients, 20 colorectal adenoma patients, 6 hyperplastic polyps patients and 102 colonoscopy-negative controls were recruited. The size of all 20 adenoma exceeds 10 mm (10–55 mm) and the median size was 20 mm. And all signed a written informed consent. The final diagnosis of patients was determined based on histopathological analysis by an experienced pathologist, and neoplasm stagings were defined according to the American Joint Committee on Cancer (AJCC) staging system as revised in 2010 (7th edition). All the patients and normal controls were divided into two groups, high-risk group (colorectal cancer and colorectal adenoma patients,same as colorectal neoplasms) and low-risk group (hyperplastic polyps patients and colonoscopy-negative controls). Meanwhile, clinical information were also collected, especially the results of serum biomarkers detection.

DNA extraction from stool samples

About 5 g of stool samples was collected from all the newly diagnosed patients and normal controls before bowel preparation by multipoint sampling which takes small aliquots from different points of the same stool sample of each patient, and then mixed it with 15 ml preservative buffer. All buffered stools were immediately transported to our laboratory and stored at − 80 °C. Before extracting genomic DNA, buffered stools were thawed and centrifuged, and then the stool sediment was used to extract stool genomic DNA by QIAamp Fast DNA Stool Mini Kit. Lysis conditions in this protocol were optimized to increase the ratio of human DNA to nonhuman DNA. The purity and concentration of extracted DNA were measured by Qubit and NanoDrop as with its A260/280 in scope of compliance (1.8–2.0). And at least, 6 μg of qualified DNA was needed for the following detection. Importantly, all assay operators were blinded to the patients' status and group. This was also applied to the following assays.

Methylation detection in stool DNA

Five micrograms of DNA was bisulfite treated using the EZ DNA Methylation Kit (Zymo Research) according to manufacturer's instructions. MethyLight (Eads CA 2000) was chosen to detect methylation of BMP3, NDRG4, SDC2 and SFRP2 with GAPDH as the internal reference by singleplex PCR. And two standards, CpGenome Universal Methylated DNA (S7821) (methylation positive) and Unmethylated DNA (S7822) (methylation negative) purchased from Merck company, were also bisulfite treated and detected as process monitoring. Primers and probes used for methylation detection were designed in the CpG island of methylated genes by Beacon Designer 8, and biosynthesis was implemented by Life Technologies. Real-time fluorescent quantitative PCR was carried out in a volume of 35 μl reaction system, which containing 17.5 μl TaqMan Universal Master Mix II (no UNG), 2.5 μl reaction mixture (primer, probe, RNase-Free ddH_2O) and 15 μl bisulfite-treated DNA. As a result, Ct value of reference GAPDH should be lower than 35, which indicated there was sufficient human DNA for analysis, and Ct value of biomarkers lower than 42 indicated a positive result.

KRAS mutations detection in stool DNA

Quantitative AS-PCR (allele-specific PCR) was chosen to detect mutations of exon 2 codons 12 and 13 (G12C, G12S, G12R, G12V, G12D, G12A, G13D) in KRAS. Primers and probes sequences were quoted from literature (Orue and Rieber 2016) and biosynthesis was implemented by Life Technologies. Positive control was purchased from Horizon. Real-time fluorescent quantitative PCR was carried out in a volume of 15.5 μl reaction system, which containing 7.5 μl TaqMan Universal Master Mix II (with UNG), 6 μl reaction mixture (primer, probe, RNase-Free ddH_2O) and 2 μl DNA (i.e. 80 ng). As a result, Ct value of reference ACTB should be lower than 28.5, which indicated there was sufficient human DNA for analysis, and Ct value of KRAS mutations lower than 36 indicated a positive result.

Immunoassays

Hemoglobin in stool samples was detected by Fecal Occult Blood Test Kit (colloidal gold) according to manufacturer's instructions. Meanwhile, blood samples were collected from patients at the first affiliated hospital of Soochow University and serum were prepared according to a routinely used standard protocol. Levels of several serum biomarkers (AFP, CA125, CA153, CA199, CA72-4, CEA, CYFRA211, NSE, SCCA, SF, tPSA, TSGF) were determined by immunoassays and platform with relevant calibrator set and PreciControl tumor marker standards for quality control. All these markers determinations were carried out by experienced clinical laboratory technicians.

Statistical analysis

A logistic regression model was built with a single biomarker or multiple biomarkers to distinguish the high-risk

group from the low-risk group. In this process, the stepwise regression method was used to select biomarkers for the final classification model based on Akaike information criterion (AIC). To be specific, stepwise regression was a method of fitting regression models in which the selection of predictive variables was carried out by an automatic procedure. In each step, a variable was considered for addition to or subtraction from the set of explanatory variables based on some pre-specified criterion, such as AIC. ROC curve was implemented to evaluate the performance of classification model built with a single biomarker and multiple biomarkers. The performance of serum biomarkers was also analyzed and compared with stool DNA biomarkers. To assess whether the results of a statistical analysis was in line with an independent data set, a validation model cross-validation was chosen to verify its fitness. In addition, the Mann–Whitney *U* test was chosen to analyze the significant difference of human DNA quantity in stool sample between high-risk individuals and low-risk individuals. GraphPad Prism 5 and *R* were used for statistical analysis and plotting.

Results

The clinical information of colorectal cancer, adenoma, polyps patients and colonoscopy-negative controls

Overall, the study comprised 105 colorectal cancer patients, 20 colorectal adenoma patients, 6 colorectal polyps patients and 102 colonoscopy-negative controls. Among these subjects, males accounted for about 53%, which showed no significant difference to females (47%). In colorectal cancer patients, patients in stage I, II, III and IV accounted for 15.2%, 31.4%, 33.3% and 12.4%, respectively. And the stage information of eight colorectal cancer patients was unavailable. Meanwhile, colorectal cancer occurred at different sites of the colorectum. Rectum was the most common place for tumorigenesis (43.8%), followed by sigmoid colon (27.6%). In addition, there were two kinds of polyps, hyperplastic polyps and adenomatous polyps, which were divided into polyps and adenomas, respectively, in this study. Specific information is shown in Table 1.

Table 1 Clinical information summary of patients and controls

Attributions	Sample size	Percentage (%)
Sex ($n=233$)		
Male	124	53.2
Female	109	46.8
Controls ($n=102$)		
Negative colonoscopy	102	100
Cases ($n=26$)		
Polyps	6	23.1
Adenoma (> 1 cm)	20	76.9
Cancer ($n=105$)		
I stage	16	15.2
II stage	33	31.4
III stage	35	33.3
IV stage	13	12.4
Unavailable	8	7.7
Cancer ($n=105$)		
Ascending colon	12	11.4
Transverse colon	3	2.9
Descending colon	4	3.8
Sigmoid colon	29	27.6
Rectum	46	43.8
Unavailable	11	10.5

The performance of single stool-based biomarker for colorectal neoplasms screening

A logistic regression model was built with Ct values of single stool-based DNA biomarker. Herein, all these samples were divided into two groups: colorectal cancer and adenoma as the high-risk group (colorectal neoplasms), polyps and colonoscope-negative as the low-risk group. Table 2 showed the performance of every biomarker in colorectal neoplasms screening, including sensitivity, specificity and accuracy, and ROC curve of every biomarker is shown in Fig. 1. Based on accuracy, all stool biomarkers could be ranked as: FIT > SDC2 > SFRP2 > KRAS > NDRG4 > BMP3. The fecal immunochemical test (FIT) showed the best performance with an accuracy of 81.6% and methylation of BMP3 performed the worst in colorectal neoplasms screening. Other than BMP3 methylation, the performance of another methylation biomarker—NDRG4, which showed good performance in an FDA-approved colorectal neoplasms screening test named Cologuard, was also poor.

The performance of a stool-based DNA biomarker panel for screening of colorectal neoplasms

By stepwise regression analysis, several tumor biomarkers were filtered, and a model was chosen by AIC. Finally, a panel composed of KRAS mutations, methylation of SDC2 and SFRP2, fecal occult blood testing and reference gene GAPDH was well behaved in the screening of colorectal cancer and adenoma. By logistic regression analysis, this panel showed a sensitivity of 91.4% (95% CI, 83.9–95.8%) for colorectal cancer and 60% (95% CI, 36.4–80.0%) for adenoma at the specificity of 86.1% (95% CI, 77.8–91.8%) (see Fig. 2a). To assess whether the results of this statistical analysis was in line with an independent data set, 3×10 cross-validation (tenfold cross-validation in three

Table 2 Sensitivity, specificity and accuracy of every biomarker in colorectal neoplasms screening

Biomarker	Sensitivity (cancer = 105) (%)	Sensitivity (adenoma = 20) (%)	Sensitivity (high risk = 125) (%)	Specificity (low risk = 108) (%)	Accuracy (total = 233) (%)
BMP3 methylation	37.1	15	33.6	89.8	59.7
NDRG4 methylation	46.7	25	43.2	90.7	65.2
SDC2 methylation	68.6	50	65.6	91.7	77.7
SFRP2 methylation	68.6	25	61.6	90.7	75.1
KRAS mutation	56.2	45	54.4	88	70
FIT	86.7	40	79.2	82.4	80.7

High-risk group, equivalent to colorectal neoplasms, was composed of colorectal cancer and adenoma in this study

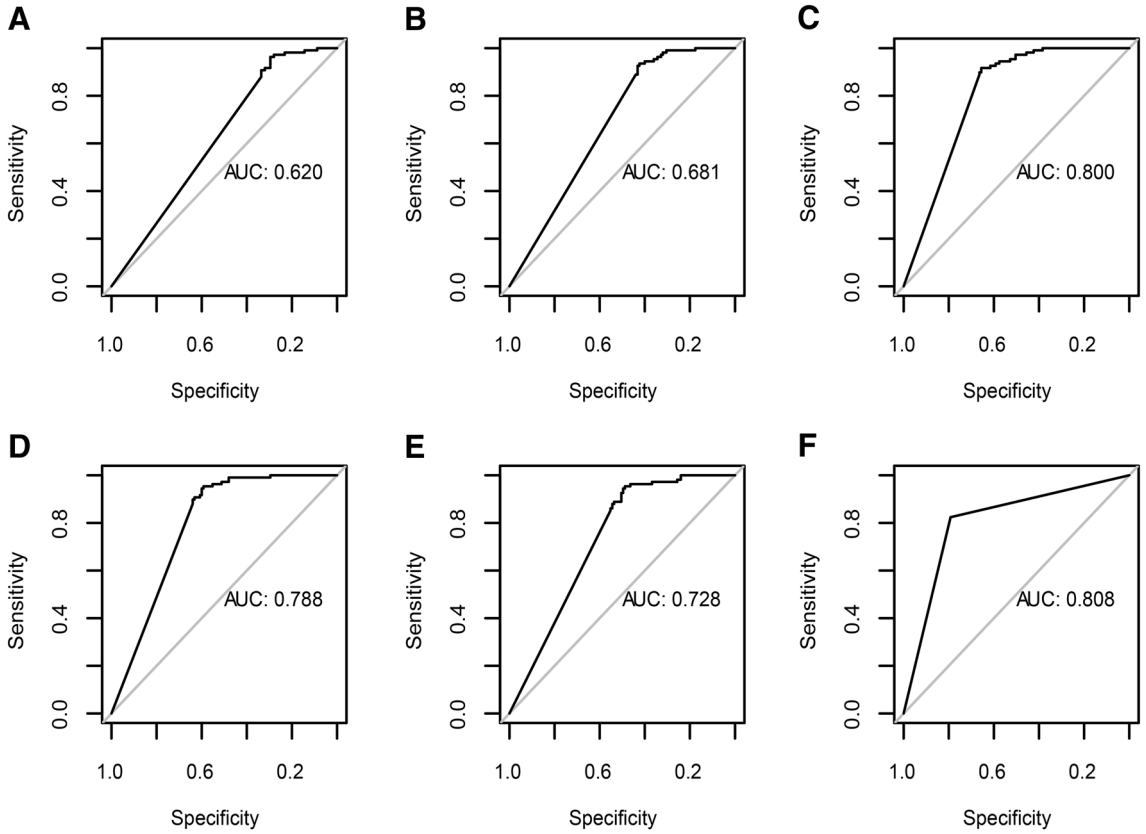

Fig. 1 ROC curves and area under the curve to evaluate the performance of every stool biomarker for distinguish high risk from low risk. **a** BMP3 methylation; **b** NDRG4 methylation; **c** SDC2 methylation; **d** SFRP2 methylation; **e** KRAS mutations; **f** hemoglobin. *X*-axis: specificity of the classifier; *Y*-axis: sensitivity of the classifier

repetitions) was implemented and the result is shown in Fig. 2b. Cross-validation was a technique used to evaluate predictive models by splitting the original sample into a training set to train the model, and a test set to evaluate it. And tenfold cross-validation meant that the original sample was randomly split into ten equal sized subsamples. Of the ten subsamples, one subsample was left as the validation data for testing the model with the other nine subsamples used as training data for training the model. This process was repeated ten times (the fold), ensuring each of the ten subsamples was used exactly once as the validation data. Herein we did tenfold cross-validation for three times. At last, the average accuracy of 3×10 cross-validation was 84%, which proved the great performance of the panel of multi-target stool-based biomarkers once again. Using the final classification model built with all samples, the sensitivity of this panel for screening of colorectal cancer at different stages is shown in Fig. 2c. For patients in early stage of colorectal cancer (I and II), the sensitivity of the panel was 87.5% and 100%, which suggested that the panel

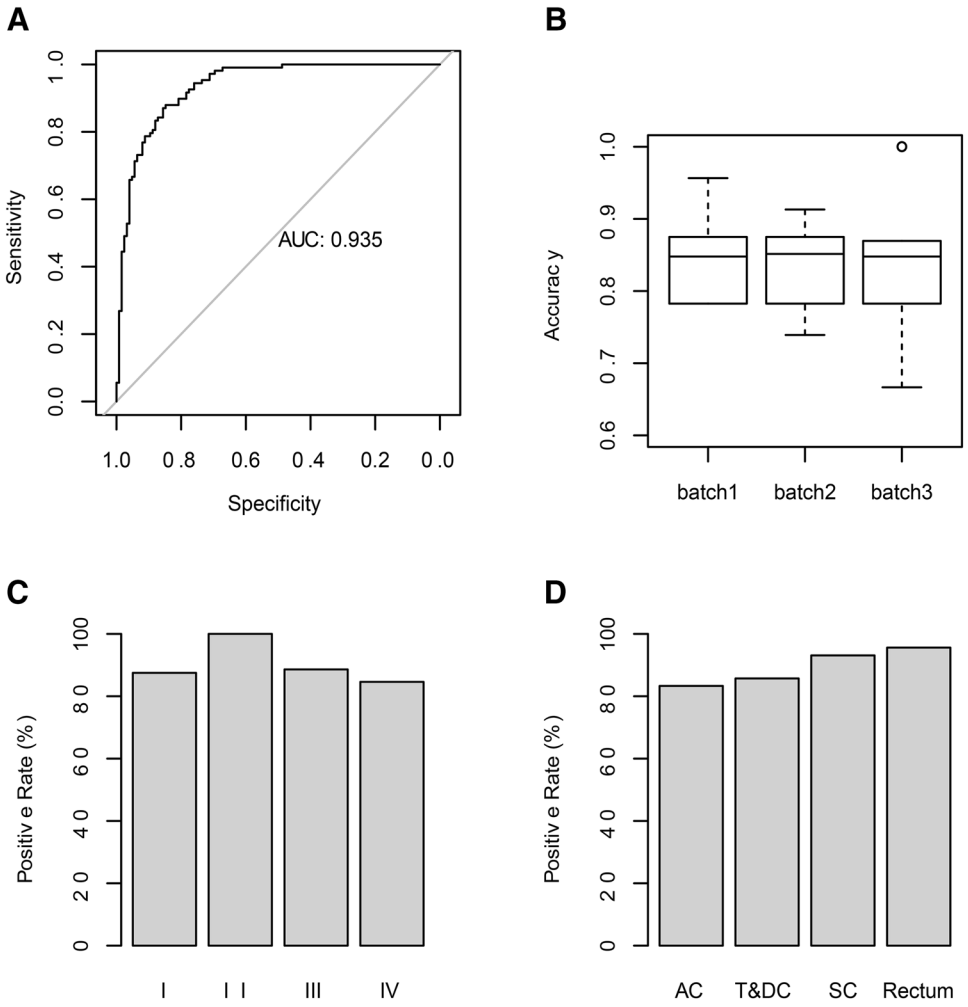

Fig. 2 The performance of multi-target stool DNA testing for detection of colorectal cancer and adenoma. **a** The ROC curve and area under the curve of multi-target stool DNA testing for colorectal cancer and adenoma screening; **b** the accuracy of 3×10 cross-validation for colorectal cancer and adenoma screening, batch 1–3 represents three repetitions; **c** the positive rate of multi-target stool DNA testing in colorectal cancer stage I (16), II (33), III (35), IV (13); **d** the positive rate of multi-target stool DNA testing in cancer from AC (ascending colon, 12), T&DC (transverse colon and descending colon, 7), SC (sigmoid colon, 29), rectum (46)

could be applied for early screening of colorectal cancer. In addition, the positive rate of cancer from ascending colon (12), transverse colon and descending colon (7), sigmoid colon (29), rectum (46) was 83.3%, 85.7%, 93.1% and 95.6% respectively (see Fig. 2d), suggesting that the panel of multi-target stool-based biomarkers was also well behaved in the screening of lesions in full length of colon and rectum. Similar to colonoscopy (Ai et al. 2018), it was less effective for screening of lesions in proximal colon than distal colon.

Comparison between multi-target stool-based biomarkers and conventional serum biomarkers in colorectal cancer screening

To compare the performance of multi-target stool-based biomarkers with conventional serum biomarkers in colorectal cancer screening, levels of multiple serum biomarkers were analyzed. Based on current criteria, the positive rate of every serum biomarker in colorectal cancer patients is shown in Fig. 3. Among these conventional serum biomarkers, both CEA and TSGF (tumor-specific growth factor) had two

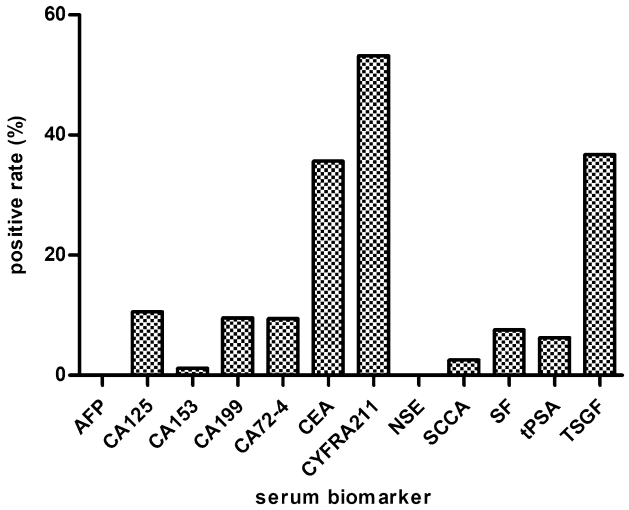

Fig. 3 Positive rate of serum biomarkers in colorectal cancer patients. *NSE* neuron-specific enolase, *SCCA* squamous cell carcinoma antigen, *SF* serum ferritin, *tPSA* total prostate-specific antigen, *TSGF* tumor-specific growth factor

classification standards. As to CEA, the positive criteria were more than 10 ng/ml for smokers and more than 5 ng/ml for non-smokers. As for TSGF, 64–71 U/ml was judged as suspect and > 71 U/ml was judged as positive. Here we adopt the standards: > 5 ng/ml and > 71 U/ml for CEA and TSGF, respectively. As a result, only CYFRA211 showed a relatively better performance with a positive rate of more than 50% in colorectal cancer patients. The positive rate of TSGF could rise to 62% if suspicion standard (64–71 U/ml) was classified into "positivity", but its specificity still needed to be validated. The positive rate of most of the conventional serum biomarkers was less than 20% in colorectal cancer patients which indicated poor sensitivity in the screening of colorectal cancer. As a comparison, a panel of multi-target stool-based DNA biomarkers performed much better than conventional serum biomarkers in screening of colorectal cancer with sensitivity of 91.4%.

Elevated human DNA in stools was also a potential tumor biomarker for colorectal neoplasms screening

According to literatures, the quantity of human DNA in stool sample also showed differences between colorectal cancer and normal controls. To explore its potential value in screening of colorectal cancer and/or adenoma, Ct values of reference genes for KRAS mutation detection (ACTB) and DNA methylation detection (GAPDH) which reflected the quantity of human DNA in stool to some extent were gathered and analyzed. The average Ct value of ACTB in high-risk individuals (colorectal cancer and adenoma) and low-risk individuals (polyps and normal controls) was 22.27 and 24.53, respectively. And the average Ct value of GAPDH in these two groups was 29.98 and 34.14, respectively. Both of them showed a significant difference between high risk and low risk. For further exploration, Mann–Whitney *U* test was implemented to analyze the difference of Ct values of reference gene in two groups. As a result, the Ct values of reference gene were significantly different in two groups with a p value < 0.001 (see Fig. 4). In summary, the quantity of human DNA in stool could also be applied for colorectal neoplasms screening.

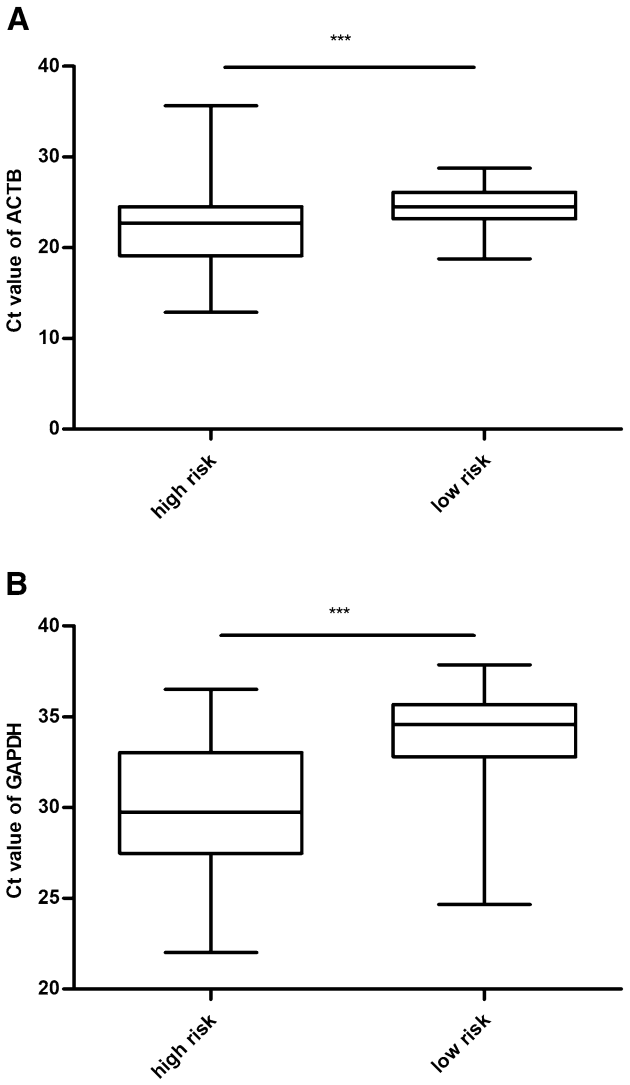

Fig. 4 Comparation of Ct values of reference genes in high-risk and low-risk individuals. **a** ACTB reference gene in detection of KRAS mutations; **b** GAPDH reference gene in detection of DNA methylation. "***" was equal to p value < 0.001 which indicated that the difference between Ct values of ACTB and GAPDH in two groups was significant

Discussion

Patients in early stage of colorectal cancer always show no obvious symptoms which make cancer screening more challenging. As with irregular medical examination, most colorectal cancer patients have been diagnosed with advanced stage and subjected to poor prognosis. Based on data between 2004 and 2010 from National Cancer Institute, the 5-year relative survival rates in stage I, II, III and IV were 92%, 76%, 71% and 12% for colon cancer and 88%, 65%, 71% and 13% for rectum cancer. There was significant difference between 5-year relative survival rate of stage I and stage IV. Meanwhile, Ann G. Zauber carried out a study on the impact of screening in incidence and mortality of colorectal cancer in 1975–2000 in America, and the results showed that screening accounted for 50% of the CRC incidence decline (22%) and 53% of the mortality reduction (26%) in the whole period (Zauber 2015). Hence, early screening was the most effective method to prevent colorectal cancer.

In recent years, non-invasive cancer screening had attracted more and more attention from the public. Several

types of biomarkers in body fluids (plasma, serum, stool) had been studied for screening of colorectal cancer and adenoma (Nguyen and Weinberg 2016), especially DNA methylation (Kim et al. 2010). With the FDA approval of Cologuard (stool DNA-based colorectal cancer screening test) and Epi proColon (blood-based qualitative colorectal cancer screening test), DNA methylation was further confirmed as an applicable biomarker for colorectal neoplasms screening.

In this study, the methylation of BMP3, NDRG4, SDC2, SFRP2 and KRAS mutations, as well as hemoglobin in stool samples (multi-target stool-based testing) were detected in colorectal cancer, adenoma, polyps patients and normal controls, and the performance of single biomarker or multiple biomarkers was then analyzed for early screening of colorectal neoplasms. When a single biomarker was used for colorectal neoplasms screening, the sensitivity ranged from 33.6% to 79.2%. Among them, the fecal occult blood test performed well, with an accuracy of 80.7%. As reported, the sensitivity of FIT for screening of colorectal cancer and advanced precancerous lesions was 73.8% and 23.8%, respectively, with a specificity of 94.9% in a study that consisted of 9989 participants (Imperiale et al. 2014). From the report, it could be seen that fecal occult blood test showed relatively high sensitivity in cancer screening, but the sensitivity was lower than 30% in screening of precancerous lesions. And it was also reported in a systematic review with meta-analysis that fecal occult blood tests showed lower colorectal cancer detection rates in the proximal colon in colonoscopy-verified diagnostic studies (Hirai et al. 2016). Compared with fecal occult blood test, a panel of multi-target stool-based biomarkers (methylation of SDC2 and SFRP2, KRAS mutations, hemoglobin) showed a sensitivity of 60% in colorectal adenoma screening, and performed much better in the screening of colorectal neoplasms in both proximal colon and distal colon. Meanwhile, the performance of panel seemed to be less affected by the location of neoplasms.

Except for fecal occult blood tests, KRAS mutations, methylation of SDC2 and SFRP2 showed an accuracy of 70%, 77.7% and 75.1%, respectively. According to literatures, 42% of colorectal cancer patients were KRAS mutations carriers (Roa et al. 2013). The main types of mutations were located in codon 12 (80.4%), including G12D (39.1%), G12V (24.2%), G12S (6.5%), G12A (4.3%), G12C (4.3%), G12R (2.1%), and codon 13(19.6%) (G13D) (Roa et al. 2013). KRAS mutations could also be detected in HPs (hyperplastic polyps) and HP/Ads (admixed hyperplastic polyps/adenoma) patients (Chan et al. 2003). Except for tissue, stool DNA could also be used for KRAS mutations. It is reported that KRAS mutations could be detected in stool samples from patients with colorectal cancer, adenomas, hyperplastic polyps, diverticulosis and hemorrhoids, as well as colonoscopy-negative controls (Battaglia et al. 2014). In our study, KRAS mutations showed a sensitivity of 54.4% at the specificity of 88% in distinguishing high-risk group from low-risk group which suggested its potential value in colorectal neoplasms screening in a Chinese population. And in concordance with previous reports, our study also confirmed that methylation of SDC2 and SFRP2 in stool DNA was a really effective biomarker for screening of colorectal neoplasms in a Chinese population.

In addition, sensitivities of KRAS assay and FIT in our study were higher than common reports (e.g., KRAS 30–40%, FIT 70–80%). For this situation, there were mainly two reasons which could explain the difference of sensitivity. First, the sensitivity rested with classification standards to some extent. If the cutoff value (standard) was raised, the sensitivity would decline along with the rise of specificity. Though the sensitivity of KRAS assay and FIT were higher in our study, the specificity was lower than 90%, especially specificity of FIT. Second, the sensitivity also rested with detection method and sample compositions. For example, the sensitivity of KRAS assay and FIT should be different between different stages of colorectal cancer. In this study, all patients were newly diagnosed and previously untreated, and patients in early stages accounted for nearly 50%, which might cause some differences. Consequently, the sensitivities of KRAS assay and FIT might be different in various studies. For example, Hershkovitz et al. analyzed the concordance of KRAS mutation in 70 tumors that contained both adenoma and carcinoma components, and finally detected KRAS mutation in 30 (43%) of the adenoma cases and 36 (51%) of the carcinoma cases (Hershkovitz et al. 2014). Chen et al. conducted a prospective clinical trial consisting of 611 subjects (25 CRC, 60 precancerous lesions), and sensitivity for detecting CRC was 96% for IFOBT with specificity of 72% (Chen et al. 2012).

Compared with single stool-based biomarker, a panel of them performed much better with a sensitivity of 91.4% and 60% for screening of colorectal cancer and adenoma at specificity of 86.1%. In logistic regression analysis, two previously reported effective biomarkers—methylation of BMP3 and NDRG4 were abandoned by stepwise regression as with lower accuracy, and the final classification model was built with four biomarkers—methylation of SDC2 and SFRP2, KRAS mutations, hemoglobin. And the relatively weaker performance of methylation of BMP3 and NDRG4 might be associated with the effect of different hereditary backgrounds of cancer in different races. Finally, our study proved the panel of methylation of SDC2 and SFRP2, KRAS mutations and hemoglobin to be effective in improving screening of colorectal cancer and adenoma in the Chinese.

In this study, we also compared the performance of a multi-target stool-based biomarker panel with serum biomarkers, and a statistical analysis was carried out. As a

result, most serum biomarkers performed unsatisfactory, with a positive rate of lower than 20% in colorectal cancer patients, which was significantly lower than stool-based biomarkers.

In addition, we found that the quantity of human DNA in stools was significantly higher in the high-risk group (colorectal cancer and adenoma) than in low-risk group (polyps and normal controls). It was reported that average concentration of human DNA was 100 ng/g, which accounted for roughly 0.01% of total stool DNA (Klaassen et al. 2003). For colorectal cancer patients, the quantity of human DNA in stools was elevated. By quantitative determination of human DNA in stools, the sensitivity for screening of colorectal cancer was 43–57% at a specificity of 97% (Osborn and Ahlquist 2005), suggesting human DNA quantity in stools to be a potential tumor biomarker in colorectal cancer screening. And the quantity of human DNA was determined by the quantity of intestine cells dropped into metabolic wastes, so it was influenced by several factors especially the stage of neoplasms. Thus, more exploration needs to be carried out to confirm it.

In conclusion, our study verified the application of a panel of stool-based biomarkers in colorectal cancer and adenoma screening and constructed a classification model with selected biomarkers which showed a sensitivity of 91.4% for colorectal cancer and 60% for adenoma with specificity of 86.1%. Compared with conventional serum biomarkers and fecal occult blood test, a panel of multi-target stool-based DNA biomarkers was a more suitable and effective screening method for colorectal neoplasms. Considering the non-invasiveness and high accuracy, it was more prone to be applied for colorectal cancer screening in asymptomatic persons at average risk for colorectal cancer. Nonetheless, the shortcoming of the study is that we did not possess another set of stool samples for the validation of the classification model. A well-designed prospective study could be conducted in the future to assess the ability of classification model in early screening of colorectal cancer.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval All procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and with Declaration of Helsinki.

Informed consent Informed consent was obtained from all individual participants included in the study.

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