Long-fragment DNA as a potential marker for stool-based detection of colorectal cancer

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1. Abstract

As neoplasia cells exfoliated from colorectal epithelium have dysfunctional apoptotic mechanisms, it is possible to identify high-molecular weight DNA fragments (long DNA) in feces. We performed this study to evaluate the sensitivity and the specificity of fecal-based long DNA assay for colorectal cancer (CRC) detection. Feces were collected from 130 patients with CRC prior to surgical treatment and 54 healthy volunteers. Presence of long DNA (APC, KRAS, BRAF, Tp53) was assessed by PCR followed by electrophoresis. We found long DNA occurrence in feces with a sensitivity of 56.2% and a specificity of 96.3% for CRC detection. In addition, long DNA was found in 58/90 (64.4%) distal CRC and 15/40 (37.5%) proximal CRC. This study illustrates the potential of fecal long DNA assay by a non-invasive and easy-to-perform in detecting individuals with CRC.

2. Background

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality in male and the second in female, and the fourth mortality from cancer in the worldwide. It was estimated 1.2 million new cancer cases and 608,700 CRC related deaths were occurred in 2008 [1]. As more than 95% of cases of CRC cases would have benefit from curative surgery if diagnosed at an earlier stage or precancerous stage [2], it is important to develop high sensitive and specific assay with a non-invasive, low-cost, and easy-to-perform method to detect early stage of CRC. To date, a large amount of elevated methods have developed for early detection of CRCs, including endoscopic examinations, blood- and stool-based tests [2]. Colonoscopy is regarded as a gold standard for its capacity to remove precancerous and cancerous lesions. However, because of its invasiveness, patient's compliance is poor. Although various blood tests using protein, cytological, microRNA, and DNA markers have been investigated, most of them were not pertinent to the clinical applications [3]. The main approach of CRC screening is the fecal occult blood test (FOBT) in the world [3]. However, the sensitivity of FOBT for CRC diagnosis is only 26% for carcinoma and 12% for advanced adenoma detection [4]. Furthermore, FOBT also contains the risk of false-positive in the patients who have hemorrhoids, ulcer and inflammatory bowel disease [5-7]. To avoid FOBT disadvantages, more sensitive and specific methods has been strongly required. A variety of fecal molecular markers including mutations of oncogenes and tumor suppressor genes, microsatellite instability, mircoRNA, and DNA methylation reportedly could increase the sensitivity in the CRC screening [8-14] . In particular, fecal long DNA assay seems to be a valid and reliable method for the detection of CRC [15-19]. Long DNA is DNA from cancerous or precancerous cell, shed from dysplastic mucosa, which maintain longer fragment DNA form because of the resistance to apoptotic process [20]. On the other hand, in the cases of the apoptotic process, DNA is cleaved and 200bp DNA fragment is yielded [21]. Although various advanced technologies have applied in elevation with stool long DNA markers, time-consuming and expensive methods adverse to screening promotion.

3. Objective

This study illustrates the potential of fecal long DNA assay by a non-invasive and easy-to-perform in detecting individuals with CRC. We performed long DNA assay with a simple method using PCR and electrophoresis and found that the combination of different long DNA

markers could increase the diagnostic performance for CRC detection.

4. Materials and methods

4.1 Materials

We collected stools from 130 patients with CRC prior to surgical treatment and 54 healthy volunteers. The clinicopathologic features of the patients are shown in Table 1. The mean age of the patients was 68 year (range 37-94); 84 were males and 46 were females; 8 were Stage 0, 47 were Stage I, 24 were Stage II, 33 were Stage III, 18 were Stage IV; 40 were right-sided and 90 were left-sided or rectal. The study protocol was approved by the institutional review board and informed consent was obtained from each patient and volunteers.

Table 1 Clinical paramaters of patients with colorectal cancer

Total *n* patient		130
Sex		
Male		84
Female		46
Mean age (range)		68.1 (37-94) years
TNM stage		
	0 (Tis)	8
	I	47
	II	24
	III	33
	IV	18
Tumor site		
	Ascending colon	27
	Transverse colon	13
	Descending colon	9
	Sigmoid colon	43
	Rectum	38

4.2 Methods

4.2.1 DNA extraction

Fecal specimens were collected in Yamaguchi University Hospital and were stored at -20°C until DNA extraction. Fecal samples were thawed from -20°C and 100 - 200 mg of fecal samples were used for DNA extraction. The DNA was extracted using QIAamp DNA Stool Mini kit (QIAGEN) according to manufacturer's instructions. Extracted DNA was diluted to final concentration of 20 ng/μl.

4.2.2 PCR

We performed PCR of 4 different genes including APC, BRAF, KRAS, and p53 for the stool long DNA tests. The PCR reaction was performed with 40 ng of DNA, 1×Buffer II (Applied Biosystems), 1.5 mM MgCl2, 0.8 mM dNTPS, 1 μM of each primer, 3% DMSO, 0.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a total volume of 10 μl. The primers were shown in Table 2. Cycling conditions were as followed : preheating at 95° C for 7 min followed by 45 cycles of denaturation at 95° C for 45 s, annealing at 60° C for 30 s, and extension at 72° C for 1 min. β-actin was amplified as an internal control. PCR products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.

Table 2 Primers list for PCR (polymerase chain reaction)

Gene for L-DNA detection	Primer sets	Primer Tm (°C)	bp
APC	FOR: 5'-TATGCGTGTCAACTGCCATC-3'	63.2	800
	REV: 5'-CTCTGTTTTGGCGACGTCTA-3'	63.8	
KRAS	FOR: 5'-AGACTTGGGAGTCTTCGATCC-3'	63.3	800
	REV: 5'-CTTACTGGCACCTAGGTTAG-3'	64.0	
BRAF	FOR: 5'-CCATAGCATGAAGGCAGGTT-3'	63.8	800
	REV: 5'-CGTGTCGGTTTCAATCACGT-3'	63.2	
Tp53	FOR: 5'-TCACCATCGCTATCTGAGCA-3'	64.7	800
	REV: 5'-AAACCCTGTCCTCAGTCTCTAG-3'	63.8	
Beta Actin	FOR: 5'-TCATCTTCTCGCGGTTGGC-3'	68.8	103
	REV: 5'-CGGTTGGCGCTCTTCTACT-3'	66.9	

4.3 Statistical analysis

Statistical analysis was performed with statistical software GraphPad Prism 6 and GraphPad

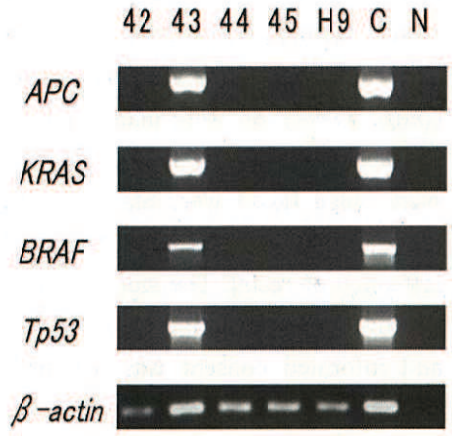
InStat 3 (GraphPad Software, La Jolla, CA). To compare variables, Fisher's test was used and a P value less than 0.05 was considered statistically significance.

5. Results

5.1 Long DNA

Figure 1 shows a representative result of long DNA assays. The frequency of long DNA of the 4 genes was significantly higher in CRC than in controls (Table 3). APC long DNA was found in 60/130 (46.2%) of CRC and in 1/54 (1.9%) of controls (P < 0.0001). KRAS long DNA was found in 50/130 (38.5%) of CRC and in 1/54 (1.9%) of controls (P < 0.0001). BRAF long DNA was found in 51/130 (39.2%) of CRC and in 0/54 (0.0%) of controls (P < 0.0001). TP53 long DNA was found in 44/130 (33.8%) of CRC and in 0/54 (0.0%) of controls (P < 0.0001). Furthermore, a combination of the 4 genes resulted in a sensitivity of 56.2% and a specificity of 96.3% for CRC screening (P<0.0001).

Figure 1



5.2 Relations between long DNA and clinicopathologic features

Table 3 shows the association between long DNA and clinicopathologic features. There was no relations of long DNA with sex or tumor stage. The frequency of long DNA was significantly higher in distal CRC than in proximal CRC. The frequency of APC long DNA was found in 49/90 (54.4%) distal CRC and in 11/40 (27.5%) proximal CRC (P = 0.0072). KRAS long DNA was found in 43/90 (47.8%) distal CRC and in 7/40 (17.5%) proximal CRC (P = 0.0010). BRAF long DNA was found in 43/90 (47.8%) distal CRC and in 8/40 (20%) proximal CRC (P = 0.0033). TP53 long DNA was found in 37/90 (41.1%) distal CRC and in 7/40 (17.5%) proximal CRC (P

Table 3 Biomarkers detectable rate in CRC compared with the patients clinic-pathological features and tumor phenotype

Samples/Biomarkers (*n*)	*APC*	*KRAS*	*BRAF*	*TP53*	Biomarker panel
CRC patients (130)	60 (46.2%)	50 (38.5%)	51 (39.2%)	44 (33.8%)	73 (56.2%)
Controls (54)	1 (1.9%)	1 (1.9%)	0 (0%)	0 (0%)	2 (3.7%)
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Sex					
Male (84)	42 (50.0%)	33 (39.3%)	35 (41.7%)	30 (35.7%)	50 (59.5%)
Female (46)	18 (39.1%)	17 (37.0%)	16 (34.8%)	14 (30.4%)	23 (50.0%)
P value	NS.	NS.	NS.	NS.	NS.
Tumor site					
Proximal (40)	11 (27.5%)	7 (17.5%)	8 (20.0%)	7 (17.5%)	15 (37.5%)
Distal (90)	49 (54.4%)	43 (47.8%)	43 (47.8%)	37 (41.1%)	58 (64.4%)
P value	0.0072	0.0010	0.0033	0.0093	0.0069
TNM Stage					
0 (Tis) (8)	3 (37.5%)	2 (25.0%)	2 (25.0%)	2 (25.0%)	3 (37.5%)
I (47)	23 (48.9%)	21(44.7%)	21(44.7%)	16 (34.0%)	29 (61.7%)
II (24)	10 (41.7%)	9 (37.5%)	11 (48.5%)	10 (41.7%)	12 (50.0%)
III (33)	13 (39.4%)	11 (33.3%)	12 (36.4%)	9 (27.3%)	16 (48.5%)
IV (18)	11 (61.1%)	7 (38.9%)	5 (27.8%)	7 (38.9%)	13 (72.2%)
P value	NS.	NS.	NS.	NS.	NS.

Pearson's chi-square and Fisher's exact tests were used to calculate *P*-values.

= 0.0093).

6. Discussion

In the current study, we showed the usefulness of the fecal long DNA as a potential marker for CRC screening. It is considered that longer template DNA is an epigenetic phenomenon consistent with the known abrogation of apoptosis that occurs with CRC [20, 22]. There appears to be abundant exfoliation of nonapoptotic cells from neoplasms [23]; in contrast, colonocyte shedding from normal mucosa is relatively sparse, and sloughed cells appear to be largely apoptotic [20, 23]. Furthermore, normal cells rapidly undergo apoptosis after detachment from their basement membrane [24]. As a hallmark of apoptosis is the cleavage of DNA by endonucleases into fragments of 180–200 bp [21, 25], it follows that human DNA in normal stools would exist primarily in fragmented forms. However, stools from patients with CRC should contains nonapoototic long DNA. In the current study, we performed long DNA assay based on PCR of 800 bp length amplicon of APC, KRAS, BRAF, and P53 genes for CRC screening. These genes were chosen based on the study by Kalimutho et al. [14] and we redesigned primers for shorter amplicon size as compared with Kaminutho's (1,015 – 1,340 bp). The sensitivity of the long DNA assay with the combination of the 4 genes was increased as compared with the single gene only. Moreover, the sensitivity was higher in the distal CRC than in the proximal CRC. Effective detection of distal colon neoplasm is of paramount importance, considering that more than 70% of CRCs occur in distal colon [26]. Further optimization and validation of long DNA assay will be needed to improve the sensitivity and the specificity.

Regarding fecal long DNA detection for CRC screening, real-time Alu polymerase chain reaction (Alu-PCR) and PCR and quantitative-denaturing high performance liquid chromatography (QdHPLC-PCR) methods have been reported [14, 27]. The sensitivity of Alu-PCR was slightly lower compared with our results (44.0% versus 56.2% of sensitivity; 100.0% versus 96.3% of specificity) [27]. Regarding the QdHPLC-PCR which evaluates PCR amplicons of 4 genes including APC, KRAS, p53, and BRAF (the same genes as in our study) [14], it showed better sensitivity (78.6% versus 56%) but a slightly lower specficity (91.6% versus 96%) for CRC screening compared with the current study. Disadvantage of QdHPLC-PCR is a requirement of an expensive equipment for QdHPLC, which results in high-cost tests. In contrast, our method needs only a thermal cycler and an electrophoresis device which enable to perform the long DNA test with low cost.

7. Conclusions

The current study suggests that the detection of long DNA in stool is a valid, feasible and low cost method to identify patients with CRC, especially with distal CRC. Further examination will be needed to confirm our findings.

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