RESEARCH COMMUNICATION

Pilot Study of the Sensitivity and Specificity of the DNA Integrity Assay for Stool-based Detection of Colorectal Cancer in Malaysian Patients

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Abstract

Background: To assess the diagnostic potential of tumor-associated high molecular weight DNA in stool samples of 32 colorectal cancer (CRC) patients compared to 32 healthy Malaysian volunteers by means of polymerase chain reaction (PCR). Methods: Stool DNA was isolated and tumor-associated high molecular weight DNA (1.476 kb fragment including exons 6-9 of the p53 gene) was amplified using PCR and visualized on ethidium bromide-stained agarose gels. Results: Out of 32 CRC patients, 18 were positive for the presence of high molecular weight DNA as compared to none of the healthy individuals, resulting in an overall sensitivity of 56.3% with 100% specificity. Out of 32 patients, 23 had tumor on the left side and 9 on the right side, 16 and 2 being respectively positive. This showed that high molecular weight DNA was significantly (p = 0.022) more detectable in patients with left side tumor (69.6% vs 22.2%). Out of 32 patients, 22 had tumors larger than 1.0 cm, 18 of these (81.8%) being positive for long DNA as compared to not a single patient with tumor size smaller than 1.0 cm (p <0.001). Conclusion: We detected CRC-related high molecular weight p53 DNA in stool samples of CRC patients with an overall sensitivity of 56.3% with 100% specificity, with a strong tumor size dependence.

Keywords: Colorectal cancer - diagnosis - stool long DNA marker - polymerase chain reaction - Malaysia

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Introduction

Colorectal cancer (CRC) is a disease in which cells in the tissues of colon, rectum, or appendix exhibit uncontrollable growth. While among the most common cancers, CRC is 90% and 75% curable if detected at stage I and II respectively (Chen et al., 2005; Olson et al., 2005). Unfortunately detection of CRC is often delayed until the patient develops disease symptoms (in about 2-3 years), by which time the cancer is no longer localized and already progressed into the higher grade (Burt, 2010). Since the pre-cancerous lesions (adenomas) take couple of years or longer to finally develop into carcinomas, there is a window period during which the pre-cancerous lesions can be detected and removed. Detection of pre-cancerous lesion via mass screening of population at risk may prove effective in decreasing CRC-associated morbidity and mortality.

Colonoscopy and flexible sigmoidoscopy are proven extremely useful in CRC detection (Young et al., 2011); however, factors such as expertise, high host, invasiveness, and patient discomfort render these methods less than ideal for mass screening (Young et al., 2011). Non-invasive methods such as fecal occult blood testing (FOBT) are available but limited in their efficacy due to suboptimal sensitivity and non-specificity (Kerr et al., 2007; Ekelund et al., 2010). There is clearly a need for improved non-invasive tests that are sensitive, specific and affordable for mass screening.

In CRC, tumors shed (exofoliate) intact cancerous epithelial cells in the intestinal lumen which presents an opportunity to study the biomarkers present on and inside these cells. Increased amount of human genomic DNA is found in the stool of CRC patients as compared to the healthy individuals (Klassen et al., 2002). Furthermore, the DNA recovered from the CRC patients stool was of high molecular weight, unlike fragmented apoptotic DNA recovered from the stool samples of colonoscopy negative individuals (Boynton et al., 2003). Increased amount of high molecular weight DNA (often referred as long DNA or L-DNA) in the stool of CRC patients may result from the decreased apoptosis of the intestinal epithelial cells and/or increased exofoliation of cancerous cells from the tumor(s) (Boynton et al., 2003; Abbaszadegan et al., 2007). Therefore, the differential presence of L-DNA in the stools of CRC patients may be used as a diagnostic marker for CRC (Boynton et al., 2003; Abbaszadegan et al., 2007). The presence of the L-DNA can easily and

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reliably be detected by polymerase chain reaction (PCR), making this a sensitive, specific, and affordable CRC test for mass screening.

Exact incidence of CRC in peninsular Malaysia is not known, however, according to the data compiled by National Cancer Registry (NCR) of Ministry of Health Malaysia, CRC was the second most common cancer after breast and the first among males and second among females (NCPR CRC, 2010; Harmy et al., 2011). A total of 2866 cases were registered with NCR in 2006 and they accounted 13.2% of all cancer cases in Malaysia. The incidence of CRC increased sharply in patients aged 40 years and above (NCPR-CRC, 2010) and it is estimated that CRC among others cancers, is on the rise in Malaysia (Harmy et al., 2011). Mass screening programs are not in place though occasionally FOBT is employed but this has not gained favor among the physicians perhaps due to the shortcomings of this test (Harmy et al., 2011). In this study, we have assessed the diagnostic potential of L-DNA PCR test on a series of CRC patients and healthy volunteers.

Materials and Methods

Sample Collection: This study was approved by the research and ethics committee, School of Medical Science, Universiti Sains Malaysia. Informed consent was obtained from every subject prior to the collection of stool sample. Stool samples were collected from a total of 64 individuals, including 32 CRC patients and 32 healthy individuals.

For the CRC patients, the diagnosis was histologically confirmed and demographic as well as clinico-pathological characteristics of the patients were recorded. The stool was collected after four to five days from colonoscopy test or before bowel preparation for surgical resection in order to avoid traumatic artifacts due to bleeding or inflammation. Healthy volunteers were laboratory staff, their relatives or friends, and frequency matched by gender, age and ethnicity. All healthy donors had a negative FOBT.

The stool samples (~ 5 g) were transported from the site of collection to the laboratory on ice and DNA was isolated on the same day. Remaining stool specimens were stored at -80^{o}C in aliquots of 1 g.

DNA Extraction

QIAamp DNA Stool Mini Kit (Qiagen 51504) was employed for the extraction of total genomic DNA from the stool essentially following the protocol supplied by the manufacture. Stool (~1 g) was homogenized in 10 mL buffer ASL, and stool slurry (2 mL) was then used to extract total DNA. DNA was finally eluted in 100 uL of buffer AE (10 mM Tris·Cl; 0.5 mM EDTA, pH 9.0).

Extracted DNA was assessed for the quality by UV spectroscopy at 260/280 nm, as well as agarose gel electrophoresis and quantified using an Invitrogen Qubit fluorometer / dsDNA BR Assay Kit (Invitrogen Q32853). The purified genomic DNA was stored in smaller aliquots at -20^{o}C.

Long DNA (L-DNA) Analysis

A 1.476 kb DNA fragment including exons 6 to 9 of p53 gene was used for the L-DNA analysis. A previously reported (Béroud & Soussi, 1998) set of primers (Forward = 5' GCCTCTGATTCCTCACTGAT 3' and Revers = 5' AAGACTTAGTACCTGAAGGGT 3') was used in the PCR. The PCR reaction was consisted of 1 X PCR buffer (minus Mg++), 0.2 mM dNTPs each, 1.5 mM of $MgCl_2$, 0.5 uM of each primer, 20 ng of template DNA, 1.0 unit of Taq DNA polymerase in a total of 25 uL reaction. The PCR reagents were from Invitrogen (10342-020). The PCR conditions were: initial denaturation at 95^{o}C / 3 min followed by 35 cycles of 95^{o}C / 30 sec; 58^{o}C / 2 min; 72^{o}C / 2 min, and a final extension at 72^{o}C for 5 min in an Eppendorf Mastercycler® pro. A total of 5 uL of PCR product was electrophoresed on 0.7% agarose gel and photographed using FluorChem M Fluorescent Imaging System (ProteinSimple).

Statistical Analysis

Results are analyzed using SPSS software version 11.5. The p value was calculated using Chi-square and Fisher exact test.

Results

Long DNA (1.476 kb) spanning exon 6-9 of p53 gene was detectable in 18 out of 32 CRC patients whereas DNA extracted from the stool of healthy volunteers was negative for the L-DNA as shown in Figure 1. The sensitivity of L-DNA test was 56.35% with 100% specificity. Since the amount of template DNA may be important for the positive amplification, we tested 5, 10, 15, 20, 30, 40, and 80 ng of template genomic DNA in PCR reaction. No PCR product was detected in the reactions containing 5, and 10 ng but a clear bands of correct size were observed in reactions with 15 ng and above of genomic DNA (data not shown). All the negative DNA samples were subjected to repeated PCR reactions with higher concentrations (30, 40, and 80 ng) of genomic DNA but this did not improve the positivity of the test (data not shown).

Out of 32 patients, 23 had tumors on the left side whereas 9 patients had them on the right side. 16 out of 23 patients with left side tumor were positive for L-DNA as compared to only 2 out of 9 patients with tumors on the right side. This showed that L-DNA was significantly ($p = 0.022$) more detectable in the patients with left side tumor (69.57%) as compared to the right side tumor (22.22%).

Out of 32 patients, 22 had the tumors larger than 1.0 cm whereas 10 had the tumors smaller than 1 cm. 18 out of 22 with tumor size larger than 1.0 cm were positive for L-DNA whereas not a single patient with tumor size smaller than 1.0 cm could be detected positively. This showed that tumor size larger than 1.0 cm can be detected at high rate (81.82%) whereas tumors smaller than 1.0 cm are likely to go undetected by this method ($p < 0.001$).

The demographic and clinico-pathological data for the patients included in this study was also collected and presented in the Table 1. The mean age of the CRC patients (both males and females) was 60.56 years whereas 62.5 % were male (mean age is 61.45 years) and 37.5% were female (mean age 59.08 years). Out of 32 patients, 19 were Malay (59.375%), 9 were Chinese (28.125%),

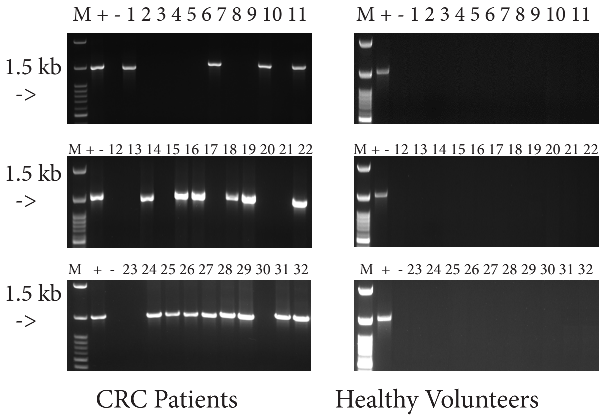

Figure 1. PCR Amplification of 1.476 kb DNA Fragment Including Exons 6 to 9 of p53 Gene. Genomic DNA was extracted from the stool samples and 25 ng was used as PCR template. For positive (+) control, 25 ng DNA extracted from a patients tumor tissue was used as PCR template. For negative (-) control, PCR-grade water was used in place of DNA template. A total of 5 uL of PCR product was loaded per well in 1.0% Agarose in TAE buffer. The gels was run at 8 volts/cm. PCR amplified products from patient (1 to 32) are shown on the left whereas data obtained for healthy volunteers (1-32) are shown on the right

and 4 were Indian (12.5%). All the tumors were invasive adenocarcinoma. The most common tumor site was rectum (40.625%) followed by sigmoid colon (21.875%), ascending colon (9.375%), recto-sigmoid colon (9.375%), descending colon (6.25%), Right hepatic flexure colon (6.25%), ascending cecum & appendix (3.125%), and transvers ascending & cecum (3.125%). Out of 32 tumors, 8 were smaller than 1 cm (25%), 10 were between 1 and 3 cm (31.25%), 8 were between 3 and 6 cm (25%), and 6 were between 7 and 14 cm (18.75%). For the tumor stages, out of 32 patients, 15 were of stage A (46.875%), 12 were of stage B (37.5%), and 5 were of stage C (15.625%) with no cases of stage D. Except for the site and the size of the tumor, no other demographic/clinic-pathological parameters correlated significantly with the positivity of the L-DNA positivity (Table 1).

Table 1. Demographic and Clinic-Pathological Characteristics of CRC Patients.

No.	Sex	Age (Yrs)	Ethnicity	Tumor Type	Tumor Site	Size	Grade	Stage	L-DNA p53
1	M	63	Mly	A.C.	R	1.4	W.D.	A	+
2	F	78	Ind	A.C.	As	3	M.D.	B	-
3	M	32	Mly	A.C.	R	0.6	P.D.	C	-
4	M	75	Ind	A.C.	As, C & AP	10	M.D.	C	-
5	M	60	Mly	A.C.	R	1	W.D.	A	-
6	F	63	Mly	A.C.	R	6	W.D.	B	+
7	F	57	Ind	A.C.	Des	0.7	M.D.	B	-
8	M	72	Chn	A.C.	R	0.6	M.D.	A	-
9	M	84	Mly	A.C.	R	2	M.D.	A	+
10	M	41	Mly	A.C.	Des	1	P.D.	B	-
11	M	57	Mly	A.C.	S	6	M.D.	C	+
12	M	37	Mly	A.C.	AS	7	M.D.	C	-
13	F	72	Chn	A.C.	RS	4	M.D.	A	+
14	F	67	Chn	A.C.	RHFC	0.8	M.D.	A	-
15	M	40	Chn	A.C.	RHFC	6	M.D.	B	+
16	F	60	Mly	A.C.	R	3.5	M.D.	B	+
17	M	60	Mly	A.C.	S	0.3	M.D.	A	-
18	M	65	Chn	A.C.	R	14	M.D.	B	+
19	M	85	Mly	A.C.	R	3.8	M.D.	B	+
20	M	60	Chn	A.C.	R	0.9	M.D.	C	-
21	M	70	Mly	A.C.	S	0.5	W.D.	B	-
22	M	78	Mly	A.C.	R	3	M.D.	A	+
23	M	51	Ind	A.C.	S	4	M.D.	C	-
24	F	44	Mly	A.C.	RS	10	M.D.	A	+
25	F	49	Mly	A.C.	S	6	M.D.	A	+
26	M	64	Chn	A.C.	T, As & C	7	M.D.	B	+
27	F	78	Chn	A.C.	S	1.5	M.D.	B	+
28	F	55	Mly	A.C.	R	7	M.D.	B	+
29	M	65	Mly	A.C.	S	3	M.D.	A	+
30	M	70	Mly	A.C.	As	0.8	M.D.	A	-
31	F	40	Mly	A.C.	RS	3	M.D.	A	+
32	F	46	Chn	A.C.	R	3	M.D.	A	+

* 'M = Male; F = Female; Mly = Malay; Ind = Indians; Chn = Chinese; A.C = Adenocarcinoma; R = Rectal; S = Sigmoid; RS = Rectosigmoid; As = Ascending; T = Transverse; Des = Descending; RHFC = Right hepatic flexure colon; C = Cecum; W.D = Well differentiated; M.D = Moderate differentiated; P.D = Poorly differentiated.

Discussion

Due to the non-invasiveness, simplicity, and moderate expertise requirements, stool DNA-based tests for the detection of CRC are highly desirable. To date, several stool DNA-based screening methods are developed, and evaluated (reviewed in Bosch et al., 2011; Kanthan et al., 2012). Some methods made use of single mutation markers whereas others included multiple mutation markers in pursuit of enhancing the sensitivity and specificity (Bosch et al., 2011; Kanthan et al., 2012). For most multi-targeted tests, the reported sensitivity was between 50 % and 75 % with specificity of 95-97 %. Multiple mutation analyses improved test sensitivity and specificity but it also increased the cost and the test execution time (Calistri et al., 2004:2009). While these tests may prove useful for research, they are not feasible for mass screening purposes. Interestingly, there were studies where DNA integrity assay (DIA) was used alone as a single marker and the sensitivity reached to 56-86 % with 81-97 % specificity (Boynton et al., 2003; Calistri et al., 2004; Calistri et al., 2009; Kalimutho et al., 2011). These studies suggest that DIA-based tests may potentially be employed for mass screening without a significant loss of sensitivity and specificity. With exception of one study (Boynton et al., 2003), the remaining three employed the use of fluorescent primers in quantitative fluorescence PCR (Calistri et al., 2004: 2009) and quantitative-denaturing high performance liquid chromatography (Kalimutho et al., 2011) which makes these tests expensive, and technically demanding.

In the present study, we isolated the DNA from the stools of 32 confirmed CRC patients and 32 age, gender, and ethnicity-matched healthy volunteers and performed a PCR for the detection of L-DNA. The aim of the study was to evaluate the sensitivity and specificity of PCR-based DIA test in a cohort of Malaysian patients for the detection of CRC. The overall sensitivity of the test was

56.35 % with 100 % specificity. However, the sensitivity of the test reaches to 81.81 % with 100 % specificity for the patients with tumor size larger than 1 cm. The size and the site of the tumor had significant correlation with the sensitivity of the test whereas other parameters such as age, gender, ethnicity, and the tumor grade had no statistically significant correlation. We postulate that this could be due to the relatively smaller sample size.

For a successful PCR reaction, the quality and amount of the template DNA is crucial. Even though we have employed a commercially available kit specifically designed for the extraction of genomic DNA from the stool samples, the quality of DNA was not comparable to the DNA recovered from the tumor tissues. Great care was taken from the transportation of the stool sample on ice to the quick processing but DNA quality couldn't be improved beyond a certain point and this was consistent for all the samples both from the CRC patients and the healthy volunteers. We tested 5 ng to 80 ng of genomic DNA as template and found 15-20 ng as the optimal concentration. Increasing the amount of template DNA did not affect the outcome of the PCR reaction for the negative results. We saw a positive correlation between the efficiency of PCR and the amount (5 ng to 80 ng) of genomic DNA isolated from the tumor tissues but higher amounts of template DNA isolated from the stool actually inhibited the PCR reaction. This suggests that the isolated DNA (using the commercial kit) was not sufficiently pure and contained PCR-inhibitory factors.

Even though any region of DNA could theoretically be used for the DIA, we have employed a PCR-primer set reported previously (Béroud et al., 1998). The PCR-primer set results in the amplification of a 1.476 kb DNA fragment spanning exon 6-9 of p53 gene. We anticipate that the primer design and the size of the resulting amplicon influence the outcome of the DIA. We have tested this possibility by comparing various genes (p53, APC, BRCA-1, BRCA-2) reported previously (Boynton et al., 2003) and found that the selection of genes (more correctly specific DNA regions), and the amplicon size does affect the outcome of the DIA at least in this cohort of patients (manuscript in preparation). We found that it was possible to improve the sensitivity (up to 65%) of the DIA without affecting the specificity (manuscript in preparation).

There is no enough data available at this time to warrant molecular-based tests as alternatives to well-established FOBT screening program (Bosch et al., 2011; Kanthan et al., 2012). Our next objective is to embark on a study with larger sample size including both, the symptomatic as well as high-risk individuals to verify the usefulness of the PCR-based DIA. Work is in progress for developing a cold-chain-free multiplex PCR reaction mix that would greatly simplify as well as standardize the PCR-based DIA and help in conducting large multi-center studies.

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