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Non-invasive detection of fecal protein kinase C β_{II} and ζ messenger RNA: putative biomarkers for colon cancer

Laurie A.Davidson, Christin M.Aymond, Yi-Hai Jiang, Nancy D.Turner, Joanne R.Lupton and Robert S.Chapkin[1]

Molecular and Cell Biology Group, Faculty of Nutrition, Texas A&M University, College Station, TX 77843–2471, USA

[1]To whom correspondence should be addressed
Email: chapkin@acs.tamu.edu

We have developed a non-invasive method utilizing feces, containing sloughed colonocytes, as a sensitive technique for detecting diagnostic colonic biomarkers. In this study, we used the rat colon carcinogenesis model to determine if changes in fecal protein kinase C (PKC) expression have predictive value in monitoring the neoplastic process. Weanling rats were injected with saline or azoxymethane (AOM) and 36 weeks later fecal samples and mucosa were collected, poly A^+ RNA isolated, and quantitative RT-PCR performed using primers to PKC β_{II} and ζ. Fecal PKC β_{II} and ζ mRNA levels were altered by the presence of a tumor, with tumor-bearing animals having a 3-fold higher ($P < 0.05$) PKC β_{II} expression as compared with animals without tumors. In addition, AOM-injection increased mucosal PKC β_{II} mRNA expression compared with saline controls. No effect of tumor incidence on mucosal PKC β_{II} expression was observed. In contrast, fecal PKC ζ expression was 2.5-fold lower ($P < 0.05$) in animals injected with azoxymethane versus saline. Since tumor incidence exerts a reciprocal effect on fecal PKC β_{II} and ζ mRNA expression, data were also expressed as the ratio between PKC β_{II} and ζ. The isozyme ratio was strongly related to tumor incidence, i.e. ratio for animals with tumors was 2.18 ± 1.25, animals without tumors was 0.50 ± 0.16, $P = 0.025$. We demonstrate that the expression of fecal PKC β_{II} and ζ may serve as a non-invasive marker for development of colon tumors. A sensitive technique for the detection of colon cancer is of importance since early diagnosis can substantially reduce mortality.

Introduction

There were an estimated 55 000 deaths from colon cancer in 1995, making colon cancer the second leading cause of death from cancer (1,2). As a large number of patients can be successfully treated if metastasis has not occurred (3,4), early detection and diagnosis are important. Therefore, an accurate technique to detect the initial changes associated with malignant transformation is essential in order to decrease mortality from colon cancer. Screening for colorectal cancer is recommended for all persons aged 50 and older with annual fecal occult blood testing or sigmoidoscopy, or both (4,5). Unfortunately, current detection methods have several drawbacks. Fecal occult blood screening can produce false positive and negative results and sigmoidoscopy is an invasive, relatively expensive procedure that only examines the distal half of the colon (4,5). Clearly, there exists the need for a test with greater specificity and higher positive predictive value (4–6).

Activation of proto-oncogenes can occur by various mechanisms, including overexpression of normal mRNA-protein product or expression of genes altered through point mutation, truncation or translocation (7). Alterations in colorectal and pancreatic tumors have been detected in the stool of patients by a non-invasive method based on the genetic pathogenesis of the disease (8–11). These protocols utilize DNA extraction procedures and detection of oncogene mutations using polymerase chain reaction (PCR*). The major disadvantage of this methodology is that it will not detect alterations in gene expression. We have developed a non-invasive method utilizing feces, containing colonocytes, in order to quantitate luminal mRNAs (12). This method is capable of isolating and quantitating specific mRNAs as candidate biomarkers in feces by incorporating the sensitivity of reverse transcriptase polymerase chain reaction (RT-PCR).

The pathogenesis of colon cancer is a multi-step process, in which tumor suppressor genes, oncogenes and other molecules involved in signal transduction are affected (13). It is now clear that signals mediated via select isozymes of protein kinase C (PKC) are involved in colonic tumor development (14–16). PKCs are a family of serine-threonine kinases thought to regulate colonic cell proliferation and differentiation. PKCs can be divided into three different sub-categories based on the cofactors needed for activation: classical PKCs (α, β_I, β_{II} and γ) require diacylglycerol (DAG) and Ca^{2+} for activation; novel PKCs (δ, θ, η and ε) are Ca^{2+} independent, but activated by DAG; and atypical PKCs (λ, ι and ζ) are Ca^{2+} and DAG independent. Although these isozymes are enzymatically similar, *in vivo*, they have different expression patterns depending on tissue and cell type (17).

PKC β_{II} protein levels are generally found in very low levels in normal rat colonic mucosa (18). However, β_{II} protein levels increase in colonic tumors as compared with normal colonic mucosa (19,20). In contrast, PKC ζ mRNA levels are significantly lower in human colorectal tumors than in normal colonic mucosa (21). PKC ζ protein levels also are lower in preneoplastic colonic epithelium from rats injected with azoxymethane (AOM) as compared with saline-injected control rats (20,22,23). Therefore, PKC β_{II} and ζ may serve as biomarkers to monitor the development of colon cancer.

In this study, a non-invasive method using exfoliated colonocytes in feces was utilized to examine alterations in PKC isozyme mRNA levels. As part of a larger study examining the effects of dietary fats and fibers on PKC isozyme mRNA levels, we report here the effect of the carcinogen AOM on mRNA levels in feces as a means of developing a non-invasive technique for the early detection of colonic tumors.

***Abbreviations:** AOM, azoxymethane; DAG, diacylglycerol; PKC, protein kinase C; RT-PCR, reverse transcription polymerase chain reaction.

Materials and methods

Animals and diet administration

The animal use protocol conformed to NIH guidelines and was approved by the University Animal Care Committee of Texas A&M University. Forty-eight male weanling Sprague–Dawley rats (Harlan Sprague–Dawley, Houston, TX) were randomly divided into eight groups (six rats/treatment) as previously described (24). Briefly, after a 1-week acclimatization period of consuming standard rat chow, rats were stratified by body wt and assigned to one of eight treatments in a 2×2×2 factorial design with two types of fat (corn oil or fish oil), two types of fiber (pectin or cellulose) and two types of injection (carcinogen or saline). Animals received the assigned diet until the conclusion of the study (36 weeks after carcinogen/saline second injection). Animals were housed individually in suspended cages in a temperature and humidity controlled animal facility with a 12 h light/dark cycle. Food and tap water were provided *ad libitum*. Forty-eight h food intakes and fecal outputs were measured during the study. Body wts were recorded weekly.

Carcinogen administration and fecal collection

After 1 week of consuming semipurified diets, rats were given two s.c. injections of AOM (Sigma Chemical Co., St Louis, MO) at a dose of 15 mg/kg body wt or an equal volume of saline (one injection/week) (24). Animals were killed by CO_2 asphyxiation 36 weeks after the second injection. The colon was subsequently removed and the most distal fecal pellet collected. The pellet was immediately placed in denaturation solution for RNA isolation (Ambion Totally RNA kit, Austin, TX). The colon was then visually inspected for tumors and tumor typing was determined (24). Briefly, tissue sections were fixed in 4% buffered formalin, embedded in paraffin, and stained with eosin and hematoxylin. Slides were then microscopically evaluated for tumors as we have previously described (24). Following removal of suspected tumors for histological evaluation, the remaining colonic sections were gently scraped with a microscope slide and the mucosa used for determination of steady-state levels of PKC isozyme mRNA. Histological evaluation of this method indicated that epithelial cells and lamina propria down to the muscularis mucosa were removed (25).

RNA isolation

Fecal and mucosal total RNA were isolated using Ambion Totally RNA kit. Fecal poly $(A)^+$ RNA was subsequently isolated using BioTecx isolation buffers (Houston, TX) and oligo dT cellulose spin columns (Collaborative Biomedical Products, Bedford, MA) as previously described (12). The fecal poly $(A)^+$ RNA pellet was resuspended in 20 µl of H_2O/0.1 mM EDTA and stored at −80°C. Quantification of fecal poly $(A)^+$ RNA was performed as previously described (12). Briefly, samples were quantitated by blotting fecal poly $(A)^+$ onto a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN). A biotinylated oligo (dT) probe (Promega, Madison, WI) was hybridized to the RNA followed by detection with streptavidin-alkaline phosphatase. Dilutions of colonic mucosal total RNA of known concentration (as determined from absorbance at 260 nm) were also blotted to generate a standard curve. For concentration calculations, it was assumed that poly $(A)^+$ RNA constitutes 3% of total RNA. Mucosal total RNA was quantitated by absorbance at 260 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) assay for negative controls (PKC γ and PKC β_I)

Aliquots of 40 ng of fecal poly $(A)^+$ RNA in a 50 µl reaction were reverse transcribed to generate first strand cDNA using Superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD) as previously described (12). PCR was performed using Expand High Fidelity polymerase (Boehringer-Mannheim, Indianapolis, IN). The 50 µl PCR reaction consisted of 1× PCR buffer, 2% DMSO, 0.05 mM dNTPs, 1.5 mM $MgCl_2$, 20 pmol each of forward and reverse primer, 2.6 U Expand High Fidelity polymerase and 5–10 µl of RT reaction. Rat brain cDNA was run as a positive control. PCR was performed using a Perkin–Elmer 2400 thermal cycler (Perkin–Elmer, Foster City, CA) with the following amplification program: 15 s denaturation (94°C), 15 s annealing (59°C), and 45 s extension (74°C) for 40 cycles. PCR products were analyzed on a 4% agarose gel followed by ethidium bromide staining. All PCR products were sequenced to ensure the fidelity of amplification (18). The primer pair for PKC γ was as follows (347 bp): forward, 5′TTG-ATGGGGAAGATGAGGAGG-3′; reverse, 5′-GAAATCAGCTTGGTCGAT-GCTG-3′. The primer pair for PKC β_I was as follows (639 bp): forward, 5′TGTGATGGAGTATGTGAACGGGGG-3′; reverse, 5′ TCGAAGTTGGA-GGTGTCTCGCTTG-3′.

Rapid competitive reverse transcription-polymerase chain reaction assay for fecal and mucosal PKC ζ and β_{II}

Rapid competitive RT-PCR was performed in order to semi-quantitatively determine the PKC ζ and β_{II} fecal and mucosal mRNA levels as previously described (26). Using this method, relative gene expression was determined by co-amplifying an exogenous DNA target ('internal standard') with a different size than the sample cDNA but with identical 5′ and 3′ ends. This allows for competition between the sample cDNA and the internal standard for primers (26). Internal standards were prepared as described previously (12). Fecal poly $(A)^+$ RNA was processed as described above. In addition, 6 µg of mucosal total RNA was reverse transcribed in a 50 µl reaction and 10 µl was amplified in the presence of either 140 fg of PKC ζ internal standard or 31.2 fg PKC β_{II} internal standard. The primer pair for the PKC ζ internal standard was (561 bp): forward, 5′CGATGGGGTGGATGGGAT-CAAAA-3′; reverse, 5′ GTATTCATGTCAGGGTTGTCTGGATTTCGGG-GGCG-3′, and for PKC ζ was (680 bp): forward, 5′CGATGGGG-TGGATGGGATCAAAA-3′; reverse, 5′-GTATTCATGTCAGGGTTGTCTG-3′. The primer pair for PKC β_{II} internal standard was (361 bp): forward, 5′-TATCTGGGATGGGGTGACAACCGAGATCATTGCTTA-3′; reverse, 5′-CGGTCGAAGTTTTCAGCGTTTC-3′. The primer pair for PKC β_{II} was (419 bp): forward, 5′-TATCTGGGATGGGGTGACAACC-3′; reverse, 5′-CGGTCGAAGTTTTCAGCGTTTC-3′. RT-PCR was performed as stated above for PKC γ with the exception that 161 fg of PKC ζ internal standard or 29.9 pg of PKC β_{II} internal standard was added to each PCR reaction. PCR products were separated on a 4% agarose gel and stained with ethidium bromide. A representative gel is shown in Figure 1. Gels were scanned and band intensities quantitated with BioImage software version 2.1 (Ann Arbor, MI). The relative amount of sample mRNA was calculated by dividing the sample band intensity by the internal standard band intensity. Specific amplification of mRNA was monitored by running PCR negative controls consisting of tubes containing either sample RNA without reverse transcription, reverse transcribed sample without mimic, or mimic only. To ensure reproducibility of results, selected samples were amplified in duplicate. In addition, the fidelity of all PCR reactions was confirmed by DNA sequencing (26).

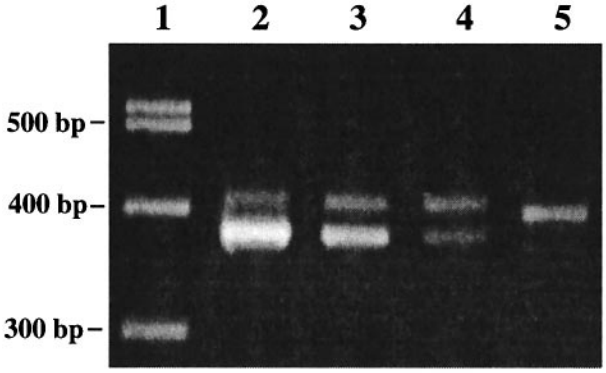

Fig. 1. Representative gel showing rapid competitive-RT-PCR of PKC β_{II}. **Lane 1**, marker; **lanes 2–5**, fecal poly $(A)^+$ samples. Upper band is the amplified sample band (419 bp), the lower band is the amplified internal standard (361 bp).

Statistical analysis

Data were analyzed to determine the effects of carcinogen and presence of tumor using one-way ANOVA. When *P*-values were <0.05 for the effects of tumor or carcinogen, total means were separated using Duncan's multiple range test.

Results

Colon carcinoma incidence

There was no evidence of carcinoma in any saline injected animal, whereas 64% of carcinogen injected rats had carcinomas at the time of death.

Effect of carcinogen and presence of tumor on fecal and mucosal PKC isozyme mRNA levels

To determine the specificity of this non-invasive procedure, PKC β_I and γ primers were used as negative controls (12,18). No amplified products were detected after 40 cycles in any fecal poly $(A)^+$ or scraped colonic mucosa total RNA samples (Figure 2). However, both isozymes were detected using brain total RNA (positive control).

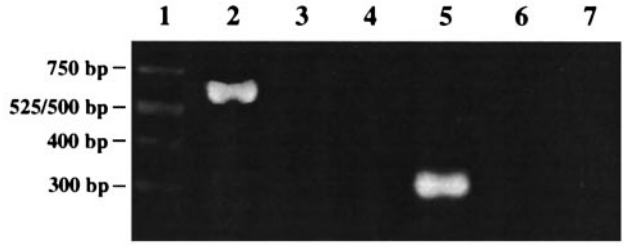

Fig. 2. RT-PCR showing expression of PKC β_I and PKC γ in brain but not in feces containing colonocytes. **Lane 1**, marker; **lane 2**, PKC β_I in brain (639 bp); **lanes 3** and **4**, PKC β_I in fecal RNA; **lane 5**, PKC γ in brain (347 bp); **lanes 6** and **7**, PKC γ in fecal RNA.

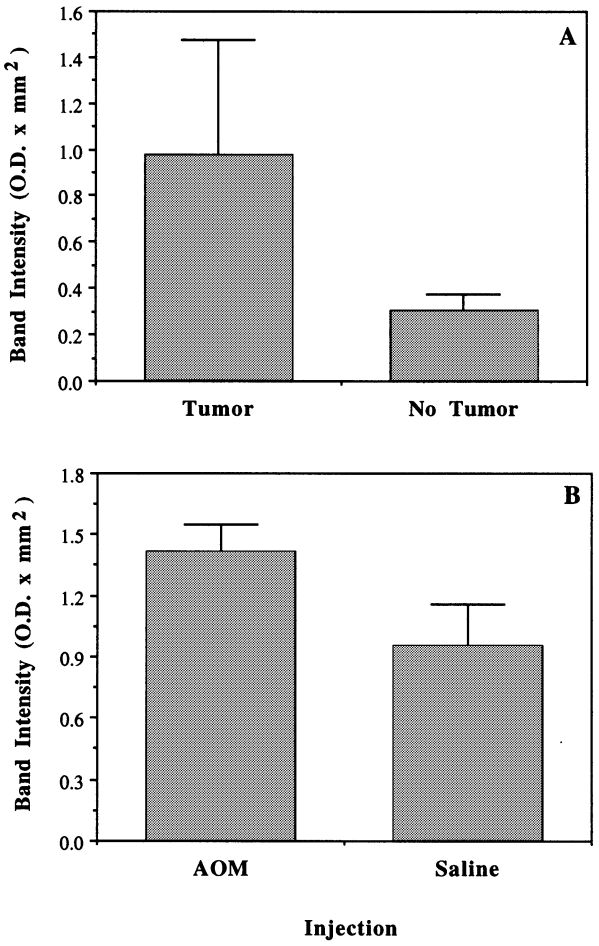

Fig. 3. Expression of PKC β_{II}. Rats were fed diets containing corn oil or fish oil and cellulose or pectin and injected with AOM or saline twice in a 2×2×2 factorial design. Feces were collected 36 weeks after the second injection and poly(A)$^+$ RNA was isolated. Colonic mucosa was scraped and total RNA was isolated. Quantitative RT-PCR was performed using primers specific for PKC β_{II}. PCR products were separated on 4% agarose gels, stained with ethidium bromide, photographed and scanned on a densitometer to quantitate. Y-axis represents band intensities (OD×mm^2). (**A**) Expression of PKC β_{II} in fecal mRNA from rats with or without tumors (mean ± SEM; $P = 0.026$; $n = 12$–29). (**B**) Expression of PKC β_{II} in mucosal RNA from rats injected with azoxymethane (AOM) or saline (mean ± SEM; $P = 0.036$; $n = 16$–20).

PCR products for PKC β_{II} were detected in all fecal and mucosal samples. Samples processed without reverse transcriptase were used as negative controls and yielded no detectable amplified products (data not shown). Using semiquantitative mimic PCR, it was determined that fecal PKC β_{II} mRNA levels were altered by the presence of a tumor,

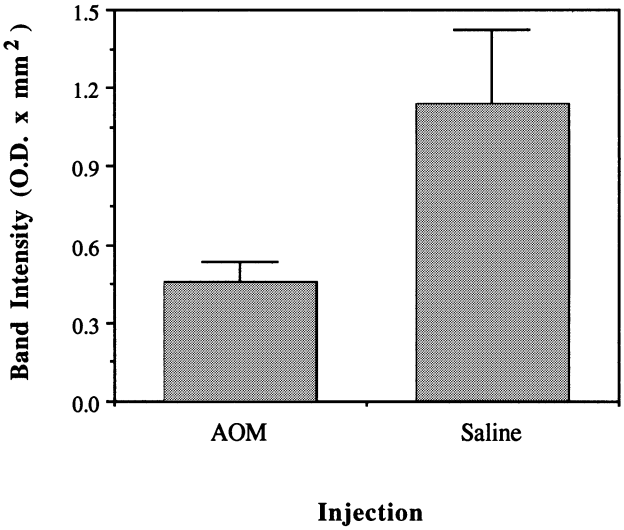

Fig. 4. Expression of PKC ζ in fecal mRNA from rats injected with azoxymethane (AOM) or saline. See Figure 3 legend for further details (mean ± SEM; $P = 0.017$; $n = 21$–22).

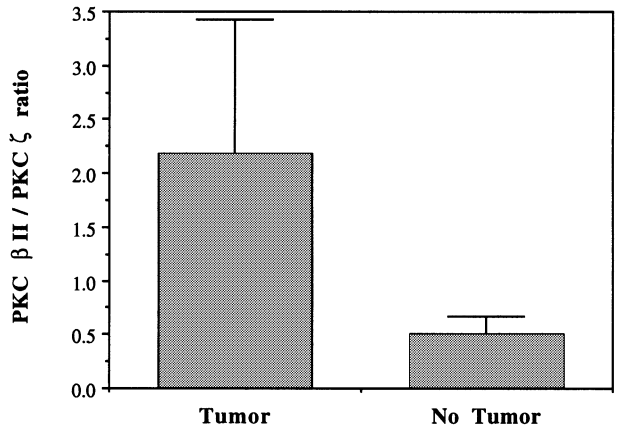

Fig. 5. Expression of PKC β_{II} /PKC ζ ratio in fecal mRNA from rats with or without tumors. See Figure 3 legend for further details (mean ± SEM; $P = 0.025$; $n = 9$–26).

with tumor-bearing animals having 3-fold higher ($P < 0.05$) PKC β_{II} expression as compared with animals without tumors, as seen in Figure 3A. In contrast, there was no effect of tumor incidence on mucosal PKC β_{II} expression. However, there was a significant effect ($P < 0.05$) of injection on mucosal PKC β_{II} expression. Specifically, carcinogen (AOM)-injection increased mucosal PKC β_{II} mRNA expression compared with saline controls (Figure 3B).

There were no treatment effects on colonic mucosal PKC ζ mRNA levels (data not shown). In contrast, fecal PKC ζ expression in rats injected with AOM was less than half ($P < 0.05$) that of saline control, as shown in Figure 4. Since tumor incidence exerts a reciprocal effect on fecal PKC ζ and PKC β_{II} mRNA expression, data were also expressed as the ratio between PKC β_{II} and PKC ζ. The isozyme ratio was strongly related to tumor incidence, i.e. ratio for animals with tumors was 2.18 ± 1.25 ($n = 9$), animals without tumors was 0.50 ± 0.16 ($n = 26$), $P = 0.025$ (Figure 5). These data demonstrate that PKC β_{II} and PKC ζ may serve as non-invasive markers for development of colon tumors.

Discussion

We have developed a novel non-invasive method utilizing feces containing exfoliated colonocytes as a sensitive technique for quantitating luminal mRNAs (12). This technique does not amplify bacterial DNA or RNA, and does not require anesthesia or cause any discomfort to the subject (12). By incorporating the sensitivity of competitive RT-PCR, we have already demonstrated that mRNA for PKC α, β_{II}, δ, ε and ζ isoforms can be detected in rat feces (12). These data are consistent with immunoblot PKC protein expression data using scraped colonic mucosa and isolated exfoliated colonocytes (18,27). In the present study, we utilized this procedure in the rat experimental colon carcinogenesis model and have identified PKC β_{II} and ζ as specific diagnostic markers, i.e. the PKC β_{II} /ζ ratio was related to tumor incidence. Carcinogen-induced changes in this ratio are consistent with previous evidence indicating that PKC may play a central role in colon carcinogenesis (14,16,17,19,20,23). Specifically, the overexpression of PKC β_{II} has been correlated with increased proliferation and blocked differentiation in colon cancer (28,29). In addition, PKC β_{II} protein levels have been shown to increase in colonic adenocarcinomas as compared with uninvolved mucosa in carcinogen injected rats (19,20). In contrast, PKC ζ protein levels are reduced in tumors (22). This isozyme may play an important role in both inhibiting tumor initiation and promotion (18,19,23), possibly by regulating colonic epithelial cell ontogeny along the crypt axis (30).

The distinct effects of carcinogen on steady-state fecal versus mucosal PKC β_{II} and ζ mRNA levels may reflect differences in the cell populations examined. Specifically, the mucosal layer was separated from the muscle layer by gentle scraping with a microscope slide. This technique removes proliferating and differentiated epithelial cells, lamina propria and muscularis mucosa (25). In contrast, mature differentiated epithelial cells are exfoliated into the fecal stream (31). It is not known whether tumor cells are a component of the population of cells in the feces. However, pre-malignant changes were detected in this study, as rats injected with carcinogen, but not yet expressing tumors, had elevated PKC β_{II} and reduced PKC ζ in exfoliated cells. Clearly, the effect of carcinogen on the precise composition of exfoliated cells should be further investigated.

Normal adult colonic epithelial cells are renewed every 3–4 days (31), resulting in the diurnal exfoliation of ~10^{10} cells (31). Recently, DNA purified from human stool samples has been utilized to detect the presence of *ras* oncogene mutations in patients with colorectal cancer (8,10,32). The major disadvantage of this methodology is that it will only detect genetic mutations. However, cancer can be caused by overexpression of mRNA from wild-type DNA, thus, measuring expression of normal or mutated genes is a more informative analytical approach. Our methodology is capable of quantifying the expression of any relevant gene by isolating and amplifying mRNA derived from sloughed colonocytes in fecal material. Although we did not use human feces, it is likely that the experimental approach described here for the rat could be utilized for the analysis of human samples. Preliminary data indicate that PKC β_{II} and ζ mRNA are found in human feces (data not shown).

Colon cancer is the second most common cause of death from cancer in the US, and early detection can result in a high cure rate (4–6). Therefore, the establishment of an accurate screening method for the disease is imperative (4). Existing methods have many drawbacks, such as fecal occult blood screening, which can produce false positive and negative results (4,6). Although adding flexible sigmoidoscopy to annual fecal occult blood increases cancer prevention 2.2-fold because of its superior ability to detect premalignant adenomatous polyps (6), it can only visualize a small segment of the colon, which is one of the reasons that molecular biology-based screening tests will likely replace current screening methodology (33). These techniques have the advantage of being able to detect early changes in the malignant transformation process, before tumor formation begins, thus making treatment more effective.

In summary, the development of non-invasive technology has the potential to provide early sensitive prognostic information and greatly enhance the current methods for cancer risk assessment. Therefore, we developed a non-invasive method utilizing feces, containing sloughed colonocytes, as a sensitive technique for detecting diagnostic colonic biomarkers. By incorporating the sensitivity of semi-quantitative RT-PCR, this novel method is capable of isolating and quantitating specific eukaryotic mRNAs as candidate biomarkers in feces. We demonstrated that the expression of fecal PKC β_{II} and ζ may serve as a marker for development of colon tumors. A sensitive technique for the detection of colon cancer is of importance since early diagnosis can substantially reduce mortality (5,6). In conclusion, non-invasive detection of exfoliated mRNA offers a powerful window into the pathobiology of the colon. Additional experiments designed to determine the suitability of PKC isozymes as predictive risk markers for human colon cancer are in progress.

Acknowledgement

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