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Quantification of human intestinal gene expression profiles using exfoliated colonocytes: a pilot study

LAURIE A. DAVIDSON[1], JOANNE R. LUPTON[1], EMIL MISKOVSKY[2], ALAN P. FIELDS[3] and ROBERT S. CHAPKIN[1]*

[1] Faculty of Nutrition and the Center for Environmental and Rural Health, Texas A&M University, College Station, TX 77843-2471, USA
[2] Department of Hepatology, University of Texas Medical Branch, Galveston, TX 77555, USA
[3] Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, TX 77555, USA

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Early detection of colon cancer can result in a high cure rate; therefore, an accurate screening method is imperative. Adoption of non-invasive testing designed to reduce anxiety over colorectal cancer screening and improve early detection is highly desirable. Therefore, we have developed a novel non-invasive methodology utilizing exfoliated colonocytes in order to quantify colonic messenger RNAs (mRNAs). Previously we have demonstrated in the rat that intact eukaryotic mRNA can be isolated due to the presence of exfoliated colonocytes in the faecal stream. To assess use of this methodology in humans, this pilot study evaluated exfoliated colonocyte mRNA expression of 11 putative biomarkers using real-time reverse transcription-polymerase chain reaction (RT-PCR) in seven normal subjects, four subjects with inflammation, and 10 tumour-bearing subjects presenting for colonoscopy. Expression of the biomarkers was evaluated following normalization to TATA box binding protein mRNA levels. Tumour-bearing subjects diagnosed with adenoma had elevated levels of cyclin Dl ($p = 0.041$). In addition, subjects displaying inflammation of the colon exhibited higher mRNA levels of cyclooxygenase-2 ($p = 0.007$). These data suggest that mRNA isolated from exfoliated colonocytes could be used to detect early stages of colon cancer, and possibly chronic inflammation. To broaden the utility of non-invasive marker analysis, additional studies are needed to generate a multi-target assay panel of diagnostic markers. This will allow for the development of robust classifiers that can determine critical gene sets for the diagnosis and prediction of colon cancer in animal models and humans.

Keywords: colon cancer, biomarkers, non-invasive, cox-2, cyclin D1

Introduction

Colon cancer is the second most common cause of cancer death in the USA and is one of the most lethal cancers (Wingo *et al*. 1995). The pathogenesis of colon cancer is a multi-step process involving mutational activation of certain oncogenes and inactivation of tumour suppressor genes, which results in uncontrolled growth and tumour formation (Fearon 1995). Despite recent advances in elucidating the molecular mechanisms that modulate the neoplastic process, the cure rate for colon cancer has not dramatically improved in the past 30 years (World Cancer Research Fund/American Institute for Cancer Research 1997, Howe *et al*. 2001). Therefore, early diagnosis of colon cancer is crucial, as a large number of patients can be cured

* Corresponding author: Robert S. Chapkin, 442 Kleberg Center, Texas A&M University, College Station, TX 77843-2471, USA. e-mail:r-chapkin@tamu.edu

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by surgical removal of the tumour if metastasis has not occurred (Levin and Bond 1996, McLeod and Murray 1999). Current detection methods are invasive and expensive (colonoscopy and sigmoidoscopy) or have a low sensitivity and specificity and can produce false-positive or false-negative results (faecal occult blood and immunohistochemical tests) (Park *et al*. 1993, Feller 1995, Mande 1997). Therefore, molecular biology-based screening tests are likely to replace current screening methods (Sidransky 1997). Markers for colorectal tumours have been detected in the stool of patients following faecal DNA analysis and detection of oncogene mutations using polymerase chain reaction (PCR) (Sidransky *et al*. 1992, Villa *et al*. 1996, Dong *et al*. 2001). However, the major disadvantage of this methodology is that it does not detect alterations in gene expression. Since activation of proto-oncogenes can occur by various mechanisms, including overexpression of normal mRNA protein products or expression of genes altered through point mutation, truncation or translocation, an mRNA-based methodology has a broader application and better predictive value in monitoring the detection of colon cancer (Sidransky 1997, Cairns and Sidransky 1999). Recently differences in gene expression have been detected at the protein level in colonocytes isolated from stool samples of asymptomatic and colorectal cancer patients (Davies *et al*. 2002).

In an attempt to improve the current screening methods, we have developed a non-invasive technique of quantifying colonic mRNAs using reverse transcription-PCR (RT-PCR) in faeces containing exfoliated colonocytes in the rat (Davidson *et al*. 1995, 1998, Chapkin *et al*. 1998). Although RNA is generally less suitable than DNA because it is readily degraded, our data demonstrated that intact eukaryotic poly A^+ RNA can be isolated because of the presence of viable exfoliated colonocytes in the faecal stream (Albaugh *et al*. 1992, Davidson *et al*. 1995). Approximately one-sixth to one-third of normal adult colonic epithelial cells are shed daily (Potten *et al*. 1979). This corresponds to the daily exfoliation of approximately 10^{10} cells. In addition, since 'global' changes in patterns of gene expression occur throughout the colon well before macroscopic tumours are apparent (Jiang *et al*. 1997, Ahlquist *et al*. 2000), these data suggest that diagnostic gene expression profiles are associated with a large number of shed cells and, hence, should not be limiting. Therefore, we have hypothesized that mRNA isolated from faeces containing exfoliated colonocytes can be used in studies for the detection of the neoplastic process in animal models and humans.

In this pilot study, we determined for the first time the feasibility of using this methodology for human colonocyte mRNA quantification. We coupled the quantification of colonocyte biomarkers using real-time PCR with a clinical diagnosis obtained by colonoscopy in order to observe markers in patients classified as 'normal' (no pathology at colonoscopy) or 'high risk' (exhibiting chronic inflammation) and in those found to have colorectal adenomas ('tumour-bearing'). Although poly A^+ RNA can be isolated directly from faecal samples immediately after defecation, acquisition at the time of colonoscopy allowed an accurate diagnosis to be obtained for each patient. Our results indicate that transcriptional gene expression profiles can be quantitatively examined using RNA isolated from exfoliated colonocytes.

Materials and methods

Human rectal vault eluate collection

The study was approved by the Human Use Review Boards at Texas A&M University and University of Texas Medical Branch, Galveston, Texas, USA. Oral and written assent was obtained from all participants, who ceased taking non-steroidal anti-inflammatory drugs and corticosteroid medication 7 days prior to colonoscopy and followed a routine bowel preparation schedule. At the time of colonoscopy, the rectal vault eluate, that is the fluid remaining in the rectum after bowel preparation for colonoscopy, was suctioned through a scope into a disposable suction trap. The trap was removed and its contents transferred immediately into lysis solution (Poly(A)Pure Kit, Ambion, Austin, Texas, USA), placed on ice until the end of the case (< 45 min) and then stored at -80°C until isolation. Samples contained very little if any faecal material, indicating good bowel preparation for colonoscopy. Patients were classified as normal (no clinical findings), inflammatory (granulomatous tissue, hyperplasia, lymphoid aggregate or inflammatory polyp) or tumour-bearing (biopsy removed at colonoscopy classified as adenoma).

Colonocyte poly A^+ RNA isolation

Colonocyte poly A^+ RNA was isolated directly from the faecal eluates using the Poly(A)Pure kit. The isolated poly A^+ RNA was subsequently treated with DNase (DNA Free, Ambion) to remove contaminating DNA. Poly A^+ RNA prepared in this manner has been shown to be free of contaminating bacterial DNA and RNA (Davidson *et al*. 1995). RNA was quantified with RiboGreen RNA quantification reagent (Molecular Probes, Eugene, Oregon, USA).

RT and real-time PCR amplification of potential biomarkers

RT was performed with 200 ng poly A^+ RNA in a 100 µl reaction using SuperScript II (Gibco BRL, Rockville, Maryland, USA). Reactions in the absence of reverse transcriptase enzyme served as negative controls. Real-time PCR was performed using the ABI 7700 (Applied Biosystems, Foster City, California, USA) and TaqMan probes. Probes and primer pairs for human genes were designed with Primer Express software and are shown in table 1. Genes were selected on the basis of studies indicating a strong correlation between their mRNA expression and colon tumour incidence, and comprised protein kinase C (PKC) beta I, beta II, zeta and iota (Davidson *et al*. 1998, Murray *et al*. 1999, Gokmen-Polar *et al*. 2001), cyclin Dl (Weinstein 1996, Arber *et al*. 1996), survivin (Kawasaki *et al*. 1998, LaCasse *et al*. 1998), cyclooxygenase type II (cox-2) (Eberhart *et al*. 1994, Kutchera *et al*. 1996), p53 (El-Mahdani *et al*. 1997), lactate dehydrogenase B (Hufton *et al*. 1999), β-catenin (Takayama *et al*. 1998) and c-myc (Hufton *et al*. 1999). Primer and probe sequences were checked for sequence homology against known genes using a BLAST search (http://www.ncbi.nlm.nih.gov/blast/). Predeveloped assay reagents (Applied Biosystems) were used to amplify p53, c-myc, β-catenin and cyclin D1. Each PCR reaction consisted of 12.5 µl 2 × universal master mix (Applied Biosystems), 300 nM final concentration forward and reverse primers, 100 nM TaqMan probe and 8 µl reverse transcription reaction in a 25 µl final reaction volume. To assess assay reproducibility, certain RT reactions were performed in triplicate followed by real-time PCR. To normalize expression levels, TATA box binding protein (TBP) was used. Expression of this marker is stable under a variety of cellular conditions and there are no known retropseudogenes that could lead to co-amplification of contaminating genomic DNA (Bieche *et al*. 1999).

Data expression

Values for the cycle threshold (C_T), that is the point at which exponential amplification of the PCR products begins to be detected, were obtained from the Primer Express software. C_T values were converted to 'expression levels' in order to allow for fold comparisons between samples using the equation

$$\text{expression level} = 2^{(40-C_T)}$$

This equation is based on the 2^n relationship of PCR amplification (i.e. the doubling of product every cycle), with 40 PCR cycles having been performed. Using the equation above, a one-cycle difference in C_T results in a two-fold difference in expression level. The expression level value was then normalized to TBP expression by dividing the marker expression level by the TBP expression level, resulting in the 'normalized marker expression level'.

Statistics

Data were analysed to determine the effect of diagnosis on marker expression by one-way analysis of variance (ANOVA).

Table 1. TaqMan probe and primer sequences used for real time PCR amplification of markers.

Marker	Probe	Forward Primer	Reverse Primer
PKC beta I	5'ctt cca gga ttc acg gtg cac atg ct	5'caa gcc aag cgt atg tat caa ttc	5'ctc taa gac aag ctt tcc aca tgt tg
PKC beta II	5'aaa ggg cat ttg gca cca ctc tct ga	5'acc ctc atc ccc aaa cta ctt g	5'gcc ttg cta gag tga ctg tgt tgt
PKC iota	5'agc agg cat cca atc atc ctt tcc ttg	5'ttg ggt aca gac aga gaa gca tgt	5'ttt ctg tct gaa agc aac aat gca
PKC zeta	5'tcg ccc ccg aaa tcc tgc g	5'tct gcg gaa ccc cga att a	5'cgg cca tca tct caa aca tg
lactate dehydrogenase b	5'ccc aga atg ctg ata gca cac gcc a	5'gtg ggt gtt gga caa gtt ggt	5'agc aag ttc atc agc cag aga ct
survivin beta	5'cac tgc ccc act gag aac gag cc	5'gcc gag gct ggc ttc at	5'aga aga aac act ggg cca agt c
cox-2	5'cat ccc tga tcc cca ggg ctc a	5'ttg tgg gaa aat tgc ttc taa gaa	5'ggg caa aga atg caa aca tca

Results

We have previously shown that poly A^+ RNA isolation from rat faecal material does not contain contaminating bacterial DNA or RNA (Davidson *et al*. 1995), which are both present in high amounts in the faeces. In this study, typical poly A^+ RNA yields from rectal vault eluates ranged from 120–1800 ng. Based on our preliminary data, significantly more poly A^+ RNA can be obtained from stool samples ($\sim$ 2–10 μg per 10 g stool). The reproducibility of the RT-PCR method is shown in figure 1. Three separate RT reactions were performed from the same starting poly A^+ sample, followed by amplification of PKC beta II and PKC zeta using real-time primers and probes. As shown in figure 1, reproducibility is excellent, with an average coefficient of variation of 0.23%. None of the reactions performed without reverse transcriptase (negative control) showed any significant amplification ($C_T \sim 40$).

The expression of the 11 markers tested in human rectal vault eluates is shown in figure 2. Data are shown for seven normal subjects, four patients with evidence of colonic inflammation, and 10 patients with tumours (adenoma). The data for the inflammatory samples were separated out from the normal patients since expression of selected markers was elevated in some patients with inflammation. Thus, while expecting to find differences between tumour-bearing and non-tumour-bearing subjects, we also found that inflammatory conditions led to differences in marker expression compared with normal subjects. For example, the average cox-2 expression was 12-fold higher in subjects with inflammation than in normal or tumour-bearing subjects ($p = 0.007$; figure 3A). A significantly elevated cyclin Dl expression (approximately seven-fold) in tumour-bearing subjects compared with normal subjects ($p = 0.041$; figure 3B) was also noted.

Discussion

We have shown for the first time in this proof-of-principle study that human colonocyte poly A^+ RNA can be isolated and biomarkers quantified by real-time PCR to determine expression levels in exfoliated colonocytes. This method has also been successfully tested on human stool samples collected soon after defecation. For example, we were able to amplify PKC beta II and zeta from poly A^+ RNA isolated from stool samples (data not shown). Therefore, although this study utilized rectal vault eluates collected by a gastroenterologist at the time of colonoscopy performed in order to obtain a clinical diagnosis, the ultimate goal is to utilize stool samples in a non-invasive context.

We initially examined genes that have been implicated in the regulation of the cell cycle (lactate dehydrogenase, cyclin Dl, c-myc, PKC zeta, beta I, beta II), apoptosis (survivin, β-catenin, p53, and PKC iota) and inflammation (cox-2). Average mRNA levels were elevated for a few of these markers in the tumour-bearing and inflammation subjects. In particular, cyclin Dl was elevated in many of the tumour-bearing patients compared with normal subjects. Increased expression of cyclin Dl is an early event in multistage colorectal carcinogenesis (Arber *et al*. 1996), emphasizing the relevance of this methodology. It is possible that the high degree of variability in expression within the tumour-bearing group could be due to stage, type or aggressiveness of the tumour. This point needs to be addressed in a much larger clinical study designed to further explore the molecular classification of colon tumour development by gene expression profiling. Indeed,

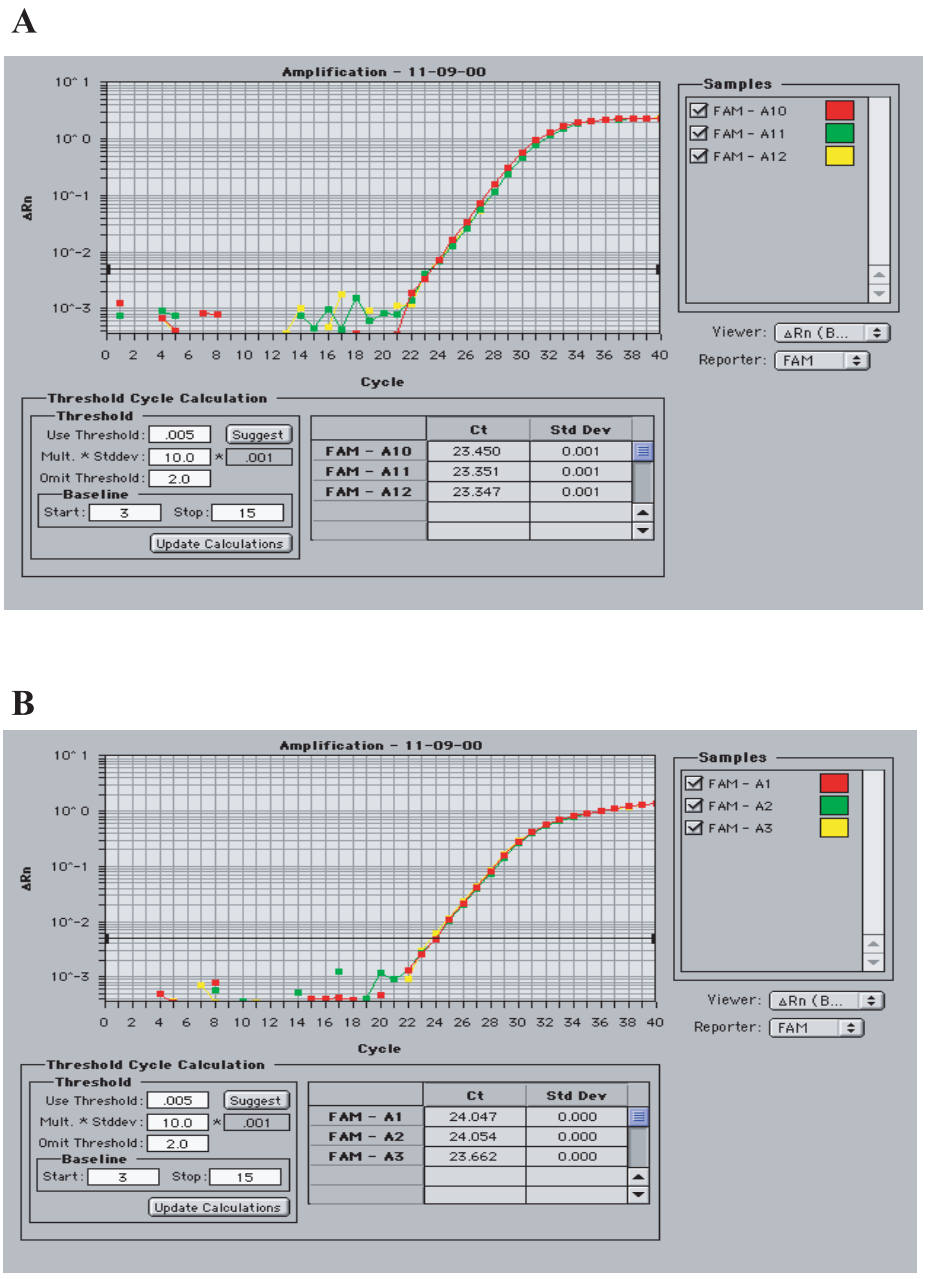

Figure 1. Reproducibility of RT and real-time PCR. RT was performed in triplicate using 200 ng poly A^+ RNA followed by real-time PCR using primers and TaqMan probes for (A) PKC beta II and (B) PKC zeta. The amplification profile showing the development of fluorescence over 40 cycles of PCR is shown. Each of the three colours is a separate RT-PCR reaction.

many of the selected markers are likely to be more telling at an earlier stage of tumorigenesis, where they may be directly involved in driving tumour formation.

With regard to colon cancer screening and the feasibility of developing a multi-target gene expression assay panel, there is concern that unless the majority of the

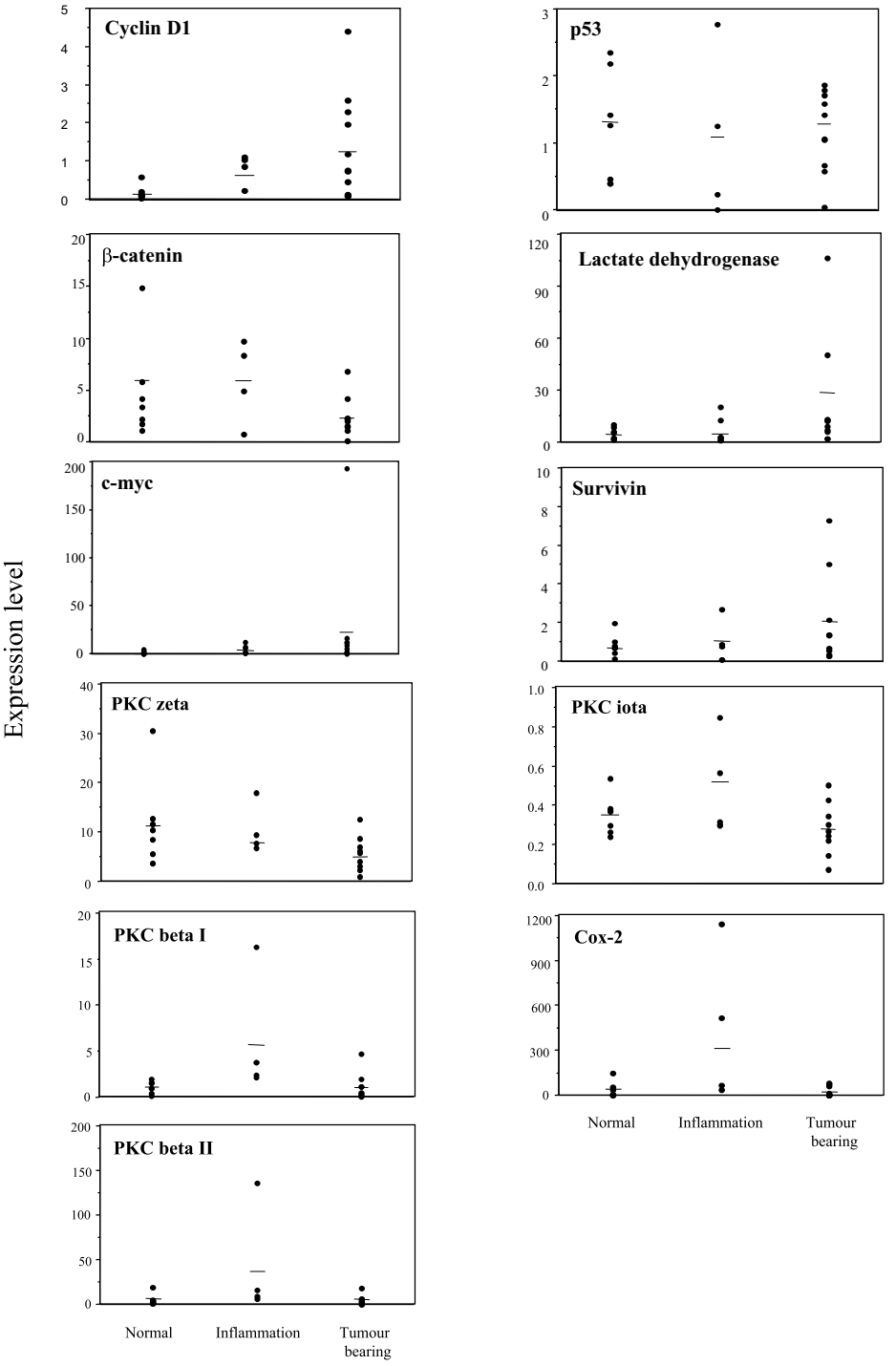

Figure 2. Expression of markers from exfoliated colonocytes quantified by real-time PCR in normal ($n = 7$), inflammation ($n = 4$) and tumour-bearing ($n = 10$) subjects. PCR was performed using primers and TaqMan probes for the 11 markers indicated. Expression levels, calculated as indicated in Materials and methods, were normalized to the expression of TBP and have arbitrary units.

shed cells are derived from a tumour, no changes in gene expression levels will be detectable against the background of normal cells. Since 'global' changes in patterns of gene expression occur throughout the colon, well before macroscopic tumours are apparent (Jiang *et al*. 1997, Ahlquist *et al*. 2000), there is evidence to suggest that 'diagnostic' gene expression profiles are associated with a large number of shed cells and, hence, should not be limiting.

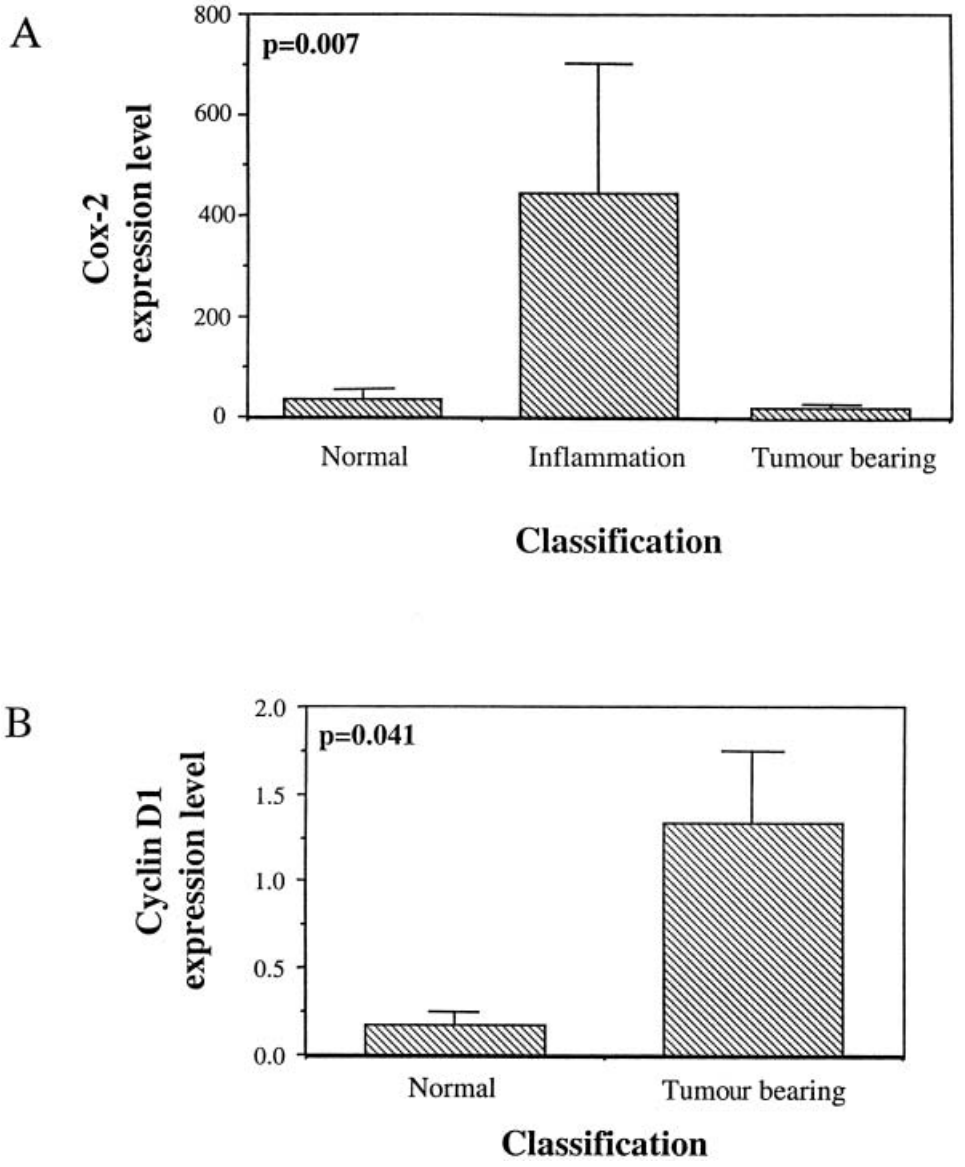

Figure 3. Expression of markers from exfoliated colonocytes quantited by real-time PCR. (A) Cox-2 expression in normal ($n = 7$), inflammation ($n = 4$) and tumour-bearing ($n = 10$) subjects was quantified by real-time PCR as described for figure 2. Average expression was significantly greater for the inflammation group ($p = 0.007$) compared with the normal and tumour-bearing subjects. (B) Average cyclin Dl expression was significantly greater in tumour-bearing subjects ($n = 10$) compared with normal subjects ($n = 7$) ($p = 0.041$).

Of the markers examined, cox-2, was elevated in patients with inflammation compared with normal subjects. These patients had active inflammatory lesions, and so elevation of this marker is not surprising, since colonic hyperproliferation is associated with epithelial cell inflammation (Biasco *et al*. 1990, Agoff *et al*. 2000). Based on these results, this methodology could be used to detect diseases of the colon other than cancer, including inflammatory conditions. Although data from only four patients with inflammatory conditions have been analysed, our results indicate the potential utility of colonocyte mRNA quantification in detecting colonic disorders.

In an attempt to broaden the observations made using real-time RT-PCR, complementary DNA/oligonucleotide microarray technology could be used for gene expression analysis of exfoliated cell poly A^+ RNA. This technology allows for large-scale high-throughput monitoring of gene expression profiles of up to 20 000 genes (Lipshutz *et al*. 1999). In addition, the molecular classification of gene expression profiling over time could be performed in order to predict experimentally verifiable phenotypic characteristics (i.e. DNA damage, aberrant crypt foci and tumours) that are relevant to colon tumour initiation, promotion and progression. Although the large majority of exfoliated cell mRNAs will be derived from the colon (Iyengar *et al*. 1991, Albaugh *et al*. 1992, Davidson *et al*. 1995), it is possible that a small percentage of cells could be derived from other organ sites. This, however, in no way diminishes the usefulness of certain mRNAs as diagnostic markers for colon cancer.

In summary, these data provide proof of principle, indicating that colorectal cancer screening by the detection of altered gene expression profiles in exfoliated colonocytes may be feasible in humans. Ultimately the goal is to identify groups of genes whose expression is altered very early in the neoplastic process, well before the development of malignant tumours. This will allow for careful non-invasive monitoring of at-risk patients through the analysis of a stool sample, so that when tumours begin to form, surgical intervention can provide a high cure rate. A much larger multi-centre clinical study would need to be performed in order to identify a panel of markers that consistently identified patients with tumours, without resulting in false-positive results from, for example, patients with inflammatory disorders. An ideal diagnostic marker would require nearly complete discrimination between normal and abnormal patients. Although the average cyclin Dl expression was significantly higher in tumour-bearing compared with normal subjects, there was an overlap between the two groups, as well as with the inflammation subjects. Therefore, on its own, cyclin Dl is not an adequate marker for tumour presence, but could provide valuable information as a component of a marker panel. The remaining markers examined in this study appear less promising, as the range of expression levels gave no discrimination between normal and tumour-bearing subjects. For this reason a large study following patients across time will be necessary to determine adequate diagnostic markers.

In conclusion, we have shown for the first time that quantification of mRNA levels from exfoliated colonocytes in humans is feasible. This novel non-invasive technology shows great promise and must be further pursued in order to identify gene expression signatures that allow the molecular classification of the earliest stages of colonic neoplasia.

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