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Molecular Markers for Human Colon Cancer in Stool and Blood Identified by RT-PCR

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Abstract. *There is a need for sensitive and specific diagnostic and prognostic molecular markers which can monitor early patterns of gene expression in non-invasive exfoliated colonocytes shed in the stool, and aggression in carcinoma cells in blood of resected colorectal cancer patients. RNA-based detection methods are more comprehensive than either DNA- or protein-based methods. By routinely and systematically being able to perform quantitative gene expression studies on non-invasive samples using carefully selected tumor-specific colon cancer genes, we can quantitatively and accurately monitor changes at various stages in the neoplastic process, allowing for surgical and/or other therapies, and thus, decrease mortality from colorectal cancer.*

Colorectal cancer (CRC) is the second and third most common malignancy in men and women, respectively, in developed countries. When diagnosed at an early localized stage, five-year survival is 90%; thus, early detection can contribute significantly to the prevention of death from this cancer (1). The most commonly used screening tests in the USA for colon adenomas in men and women, age ≥ 50 years old, are fecal occult blood testing and colonoscopy. The former test, although convenient and inexpensive and suffers from low specificity, whereas the latter one is expensive and requires cathartic preparation and patient sedation, which has resulted in a low rate of compliance (2). Computer tomography imaging of the bowl is being evaluated (3), and genetic testing for DNA mutations in colonocytes in stool are neither specific nor sensitive enough (4).

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It has been estimated that approximately 10^{10} of normal adult colonic epithelial cells, each having a lifespan of 3-4 days, are shed daily from the lower two-thirds of colon crypts (5); thus, using colonocytes to develop a screening test is an attainable goal (1, 6, 7). More recently, by employing commercial preparations, we overcame RNA's liability by stabilizing it within a short period of time after samples (*e.g.*, tissue, stool or blood) had been removed from the body, resulting in a total RNA that was readily reverse-transcribable by another commercial preparation that makes a high quality single-stranded (ss) copy (c) DNA suitable for expression profiling (8).

For detection of recurrent disease after surgical resection of the colon, patients are followed-up with history, physical examination, and laboratory tests every 3 months for 3 years (1). Although carcinoembryonic antigen (CEA) levels in plasma, after colon cancer resection, showed variations among studies and are non-conclusive, their rise automatically triggers additional imaging and endoscopy to locate resectable recurrences, and "blind" surgical exploration of the abdomen may be undertaken if necessary (9). Despite an apparently successful resection, about 10% of patients diagnosed with Dukes' A and 30% with Dukes' B colorectal tumors arising from epithelial cells relapse and die within 5 years of primary surgery, suggesting that tumor cells with metastatic potential had already escaped from the primary tumor before or at the time of resection. Adjuvant chemotherapy reduces the mortality rate by up to 22% in high-risk patients, making accurate staging essential (1). However, histopathological staging accuracy has remained largely unchanged since Dukes' original classification of CRC (10).

Recently, immunomagnetic separation combined with an RT-PCR of blood-borne epithelial cells used for detecting micrometastases was further improved by using commercial preparations and employing "unique" tissue-specific markers (11). The development and validation of molecular methods for early CRC screening, or for detection of residual occult malignancy that provides clues for aggression may better predict

diagnosis or prognosis, provide early warning of recurrence, or improve survival through timely therapy (6- 8, 11).

Materials and Methods

Adenocarcinoma cell lines and culture conditions. The adenocarcinoma cell line Caco2, which was used for validation spiking studies in normal stool and blood samples, was obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. It was propagated according to ATCC recommendations.

Acquisition of patients' clinical specimens. For this study, we employed specimens from ten control and six colon carcinoma subjects according to an approved East Carolina University (ECU) Medical Center Institutional Review Board (IRB) protocol, and all laboratory work was carried out and standardized under blind conditions and in accordance with the guidelines for handling biohazardous material that was established by ECU's Biological Safety and Hazardous Substance Committee.

Tissue specimens. A small piece of tissue sample (*i.e.*, ≤ 1 g, about 0.2 cm^3) from patients with colon adenocarcinoma was obtained from the resection margins of the operative specimens by taking mucosal biopsies from the luminal part of the bowel wall (12). Samples were processed in a sterile manner either immediately (*i.e.*, within 15 min of removal from patients to prevent degradation of RNA) and RNA extracted and stored at -70˚C for subsequent processing, or the tumor samples were flash-frozen in liquid nitrogen and stored at -70˚C until ready for enrichment by laser capture microdissection (LCM). RNA isolated from tissue was used as the "gold standard" with which to compare RNA isolated from stool or blood.

Fecal specimens. For normal specimens, a 10 g sample of feces (bowel movement) was collected from colonoscopic suctions of five individuals visiting our gastrointestinal (GI) laboratory for routine screening. Samples were inserted into sterile plastic vials, covered tightly, placed on ice for transport to the laboratory and RNA extracted immediately, followed by either RT-PCR or storage at -70˚C until further processing.

For stool specimens from adenocarcinoma subjects, the patients were identified and followed closely, so that immediately following surgical removal, the colon could be opened by the collaborating pathologist within 10 min of its removal. A 2 g stool sample was then suctioned by a disposable sterile plastic pipette and ejected into a sterile 5 ml plastic container that was placed on ice and transported aseptically to the laboratory for immediate processing (8).

Blood samples. Blood samples from three control subjects and six colon adenocarcinoma patients diagnosed histopathologically and by recurrence as stages 3 or 4 were collected in 7 ml vacutainers containing the anticoagulant lithium heparin (which does not interfere with PCR amplification), transported to the laboratory and immediately processed by differential centrifugation followed by immunomagnetic bead separation of epithelial cells (11).

Selection of cancer cells from tissue by laser capture microdissection (LCM). LCM was employed as an enrichment technique for tumors isolated from colon carcinoma patients to separate tumor cells from non-neoplastic stromal and inflammatory cells (13). The frozen colon carcinoma tissue was transported on dry ice to the Laboratory of Experimental Pathology, National Institute of Environmental Health Sciences (Research Triangle Park, North Carolina, USA) where laser capture microdissection was performed using an Arcturus PixCell II system (Arcturus Engineering, Inc., Mountain View, CA, USA), which employed a 15 μm diameter infrared (IR) laser pulse (220 mV, 49 mW) with a duration of 2.2 ms to microdissect only the tumor cells (14). Approximately 20,000 cells were captured for each preparation. The LCM samples, adhering to the thermoplastic polymer film on the plastic cap, were fitted to a 0.5 ml sterile microfuge tube, frozen on dry ice and returned to ECU for further processing (8).

Isolation of metastasized cells from blood by immunobeads capture technology. We employed the "indirect" isolation method, in which the buffy coat containing potential metastasized cells (enterocytes) was first incubated with the mono antibody (mAb) Ber-EP4 (DAKO, Glostrup, Denmark), directed against an epitope on the protein moiety of glycopeptides specifically present on the surface of human epithelial cells, and subsequently rosetted with the Dynabeads M450 sheep anti-mouse IgG (DYNAL Biotech, Inc., Lake Success, NY, USA). The rosetted Dynabeads were collected with the help of a DYNAL MPC magnet, the supernatant was pipetted off, and the Dynabeads resuspended in 10 μl fresh PBS/PSA and kept at 4˚C for 30 min until RNA extraction carried out by a commercial preparation (11).

Extraction of total RNA from LCM cells, colonocytes, enterocytes and making ss-cDNA. A commercial preparation (RNeasy Mini Kit from Qiagen, Valencia, CA, USA) was used for extraction of RNA from colonocytes in stool, carcinoma cells in circulation and enriched microdissected tumor cells in tissue of CRC patients, as described earlier (8, 11). RNA was quantitated using RiboGreen RNA quantitation reagent (Molecular Probes, Eugene, OR, USA) (15).

Another kit from Qiagen (Sensiscript Reverse Transcriptase Kit) was used as specified by the manufacturer to make high-quality ss-cDNA from < 50 ng total RNA isolated from stool, enterocytes from blood and LCM samples. We did not treat RNA with DNase I as we found this treatment to be unnecessary, and sometimes interfering with PCR amplification (16).

Extraction of total RNA from normal colon epithelium and preparing a ss-cDNA. The extraction of total RNA from mucosal epithelial cells lining the colon from normal colon tissue was carried out by homogenizing a small piece of colon mucosa (~ 0.2 cm^3) in TRI REAGENT (TR-118, from Molecular Research Center, Inc., Cincinnati, OH, USA), extracting the total RNA according to the manufacturer's specifications and storing at -20˚C in diethyl pyrocarbonate (DEPC)-treated water. Single-stranded cDNA was made as described earlier (8).

Two-step polymerase chain reaction on ss-cDNA. We used both conventional (qualitative endpoint) and real-time PCR to study the expression of selected genes in a two-step RT-PCR, as this method is preferable to the one-step RT-PCR for experiments requiring the same RT product to be used for analysis of multiple transcripts (16). Primers were prepared for: (a) the first 6 of 7 "unique" genes {PYRIN-containing APAF 1-like protein 5 (PYPAF5) [forward: 5'ACCTACCAGTTCATCGAC3'; reverse: 5' CGCTCTGAAA CCATGC 3']; H1 histone family, member 1 (H1F1) [forward: 5' CATGTCTGAAACAGTGCC 3'; reverse: 5'CTCTCCACGTCG

TAGC3']; colon and small intestine-specific cysteine-rich protein precursor (HXCP2) [forward: 5'AGGGTACATGGGCAAC 3'; reverse: 5' ACCAAA CACCGCATATT 3']; Tax 1, human T-cell leukemia virus type I binding protein 1 (TAX1BP2) [forward: 5' CACGACTTCCGTGACA 3'; reverse: 5' ACTTCGATACAC TACCATTAAC 3']; similar to olfactory receptor family 2, subfamily I, member 4 pseudogene (OR2I4P) [forward: 5' GCGACGGAGACACTAC 3'; reverse 5' GAGCGATACGA ACTTGC 3']; similar to olfactory receptor, family 2, subfamily A, member 7 (OR2A7) [forward ACACAAGATGA ATCAAGGAT AAA3'; reverse 5'GGGAACGGGACCATC3'; and found in inflammatory zone 1 (FIZZ1) [forward 5' GGGACGTTTGA TTAGATT3'; reverse 5'CCTTAGACTCCGTT ATGG3']} genes; (b) guanylyl cyclase c (GCC) gene [forward: 5' CAGGCTGTG TTCCACG 3'; reverse: 5' CCGCATCTTCCA AGTT], and (c) CEA gene [forward: 5' CCCAAACCGTCTTTT CTC 3'; reverse: 5' GCATCTTGCTTA CTGAC AT 3']. These primers were designed using Roche's LightCycler Probe Design Software, version 1.0. We also prepared primers for the β-actin that do not amplify processed pseudogenes (17) to qualitatively test amplification of this housekeeping gene in our samples before carrying out the real-time PCR analysis [forward: 5' CCTCGCCTT TGCCGATCC 3'; reverse: 5' GGA TCTTC ATGAGGTAG TCAGTC 3'].

Qualitative endpoint PCR. Qualitative endpoint PCR was carried out in an Applied Biosystem 9600 thermocycler (Foster City, CA, USA) using a master mix containing final concentrations of 1X high fidelity PCR buffer, 0.2 mM dNTP, 2 mM $MgSO_4$, 0.4 μm forward and reverse primers, 0.1 ng ss-cDNA template and 1 U of "hot start" Platinum High *Taq* DNA polymerase (Invitrogen) in a final volume of 25 μl in a 100 μl plastic PCR tube. Running conditions were: one cycle at 94˚C for 3 min to activate the hot start *Taq*, 35 cycles of 94˚C denaturation for 45 sec, 55˚C annealing for 1 min and 72˚C elongation for 1 min each, followed by one elongation/extension cycle at 72˚C for 7 min. Reactions were placed in wells of a 1% agarose gel immersed with 1X Tris-acetate EDTA (TAE) gel running buffer in an electrophoresis apparatus (5 V per cm), stained with ethidium bromide (0.25 μg/ml final concentrations), and visualized by an Alpha Innotech charge-coupled device (CCD) based imaging system (San Leandro, CA, USA).

Semi-quantitative real-time PCR. The comparative cross point (CP) method for semi-quantitative PCR analysis was employed using Roche Diagnostics real-time LightCycler (LC™)PCR instrument (Indianapolis, IN, USA), and utilized a kit from Roche (Fast Start DNA Master SYBR Green I). The DNA concentration employed was ~7 μg/ml; it was purified before being added to the reaction using the Roche High Pure PCR Product Purification Kit. In the CP method, the relative target concentration is a function of the difference between crossing points (or cycle numbers) as calculated by the Second Derivative Maximum method in which Cycler's software algorithm identifies the first turning point of the fluorescent curve in the graph showing fluorescence *vs.* cycle number. This turning point corresponds to the second derivative curve, thus explaining the name of the method. This method requires that the efficiences [$E=10^{-1/slope}$] of both target and reference gene be equal, as judged by similar slopes, and to be close to 2. For convenience, the control gene standard for RNA was β-globin in kit form obtained from Roche (LightCycler Control Kit DNA). Final primer and $MgCl_2$ concentrations were 0.5 μM and 4 mM, respectively. The

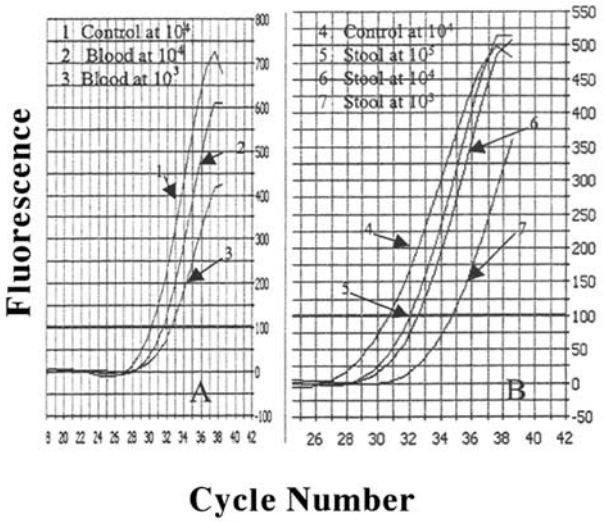

Figure 1. *Relationship between fluorescence vs. cycle number for: (A) blood having CaCo2 cells added at 10^3 (140 cells/1 ml blood) and 10^4 (1400 cells/1 ml blood), and (B) stool having cells added at 10^3, 10^4 and 10^5 cells/1 g stool, in a validation real-time RT-PCR experiment.*

DNA concentration employed was ~8 μg/ml. Control samples to exclude contamination were run in parallel with each experiment in which cDNA was replaced by water (negative control).

Statistical analysis. For practical considerations (sample size and number of observations), we compared data from three groups: (a) normal tissue (2 observations), (b) tumor tissue (4 observations), and (c) blood and stool samples (3 observations). For each of the eight genes studied, we considered the difference between the genes' crossing point (CP) to the corresponding CP value from β-globin. These differences were analyzed using a one-way analysis of variance (ANOVA) (18).

Results

Two validation studies were carried out. The first one aimed at establishing the lower limits of detection (sensitivity) of the expression of the GCC gene in Caco2 cells added to stool or blood using real-time RT-PCR. In that study, Caco2 cells were spiked and thoroughly mixed with 1 g of human stool at 10^3, 10^4 and 10^5 cells, or were inoculated and thoroughly mixed with 7 ml of normal blood at concentrations of 140 Caco2 cells/1 ml of blood and 1400 cells/1 ml of blood (each ml of blood containing 10^7 white blood cells) followed by immunomagnetic bead isolation, and real-time RT-PCR detection of the GCC gene. Each point was run in triplicate. Results are shown in Figure 1. They demonstrate that expression of the GCC gene was detected in as few as 1000 adenocarcinoma cells per gram of stool, or 140 enterocytes/10^7 WBC (8, 11). Another validation study was carried out in which 1000 Caco2 adenocarcinoma cells,

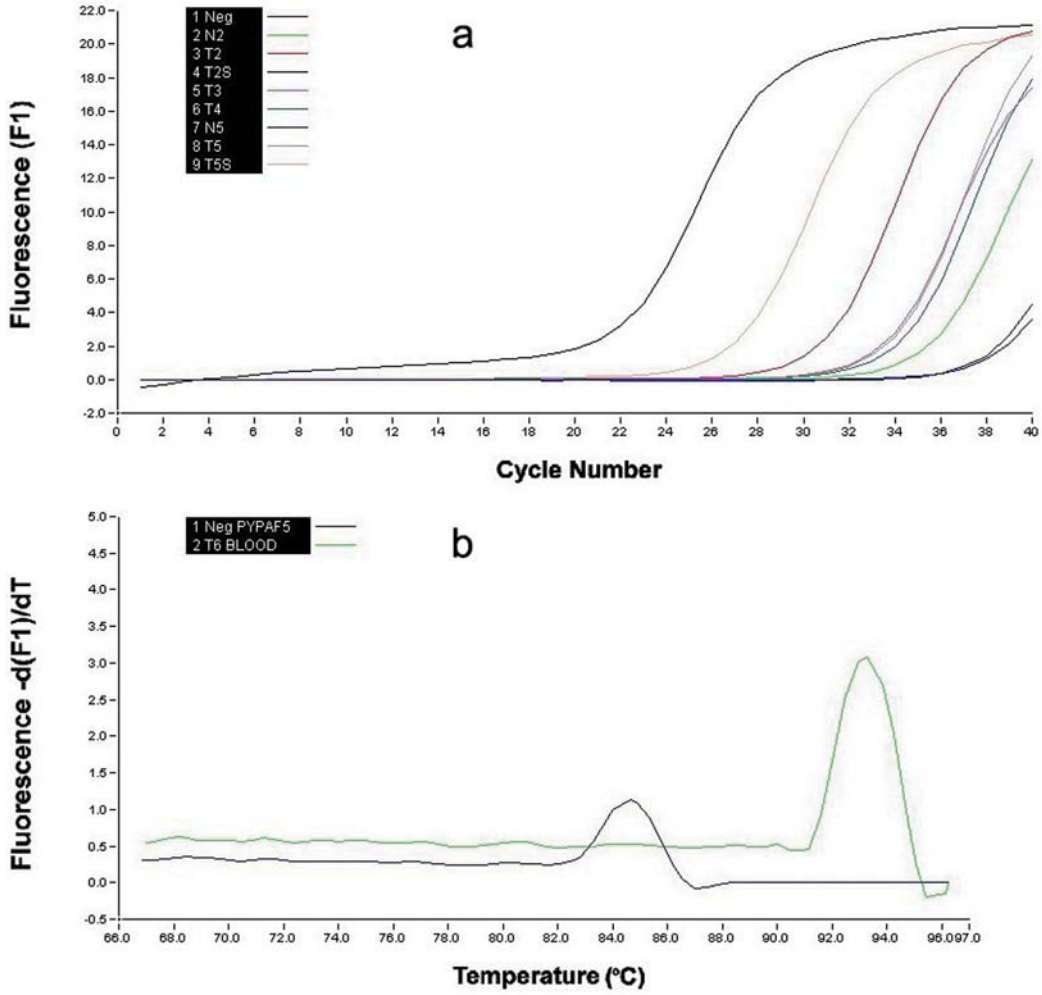

Figure 2. *(a) Relationship between fluorescent vs. cycle number for tissue/tumor-specific PYPAF5 "unique" gene in various samples using 7 μg/ml DNA. (b) Melting curve analysis for the PYPAF5 gene in blood using Roche's LightCycler™.*

incubated with mAb Ber-EP4 and Dynabeads M-450 coated with sheep anti-mouse IgG, were added to 7 ml of normal blood for various intervals (from 5 to 30 min at 4˚C) to observe and ascertain rosetting. Progressive rosetting was formed between beads and cells incubated for various time intervals; the longer the incubation, the more the resetting (11). Results are shown in Figure 1.

A third study was conducted on three separate adenocarcinoma patients to determine the amount of carcinoma cells that can be isolated from 7 ml blood by the immunoparamagnetic enrichment technique in which the final 10 ml elute-containing beads to which cells were attached was counted under a microscope. Approximately 200-400 rosetted cells per preparation (11) were used to produce enough RNA for transcription as each cell contains ~20 pg RNA (19).

The amplification specificity on all genes studied was evaluated by running 1% agarose gels on products of endpoint PCR in parallel with real-time PCR to: (a) confirm and determine the analytical specificity of the RT-PCR reaction, and (b) verify the ability of our short hybridization probes, specific for each gene studied, to bind the PCR product. We performed a conventional 25 μl qualitative endpoint PCR reaction, running 10 μl of the reaction product on an agarose gel, followed by transfer of the DNA into a Biotran™ Nylon membrane (ICN, Irvine, CA, USA) using a downward capillary transfer. After crosslinking the DNA to membranes by UV at 100 mJ/cm^2, a short hybridization probe, specific for the internal sequence of the PCR product end-labelled with digoxigenin, using terminal deoxynucleotidyl transferase (Promega Corporation, Madison, WI, USA) was prepared and hybridized. The signal was detected by chemiluminescence

Table I. *Quantification of gene expression in tissue, stool and blood from normal and colon cancer patients.*

Gene Sample	β-globin CP[a]	PYPAF5 CP	H1F1 CP	HXCP2 CP	TAX1BP2 CP	GCC CP	CEA CP	OR2A7 CP	OR2I4P CP
Neg[b]	>36.00	>36.00	>36.00	31.51	>46.00	33.88	>36.00	>46.00	36.14
N2	32.20	>36.00	32.33	>36.00	42.29	31.78	>36.00	30.78	35.96
T2	31.27	30.75	30.81	31.64	38.64	31.12	>36.00	27.86	33.08
T2 stool	31.87	22.04	21.54	24.65	29.20	23.48	25.22	23.01	24.15
T3	32.13	33.34	32.00	>36.00	39.68	32.57	>36.00	30.16	36.12
T4	30.70	35.34	30.31	31.22	39.54	32.21	34.54	29.48	34.80
N5	30.72	>36.00	>36.00	>36.00	>46.00	38.50	33.15	36.89	35.82
T5	31.13	34.05	29.43	32.66	40.52	33.17	>36.00	29.86	35.31
T5 stool	32.23	26.93	24.94	33.29	33.91	25.59	26.62	22.04	26.12
T6 blood	32.35	25.64	25.65	28.25	33.59	25.94	29.81	24.42	30.05

N=normal tissue; T=tumor tissue; N2=normal tissue from patient 2; N5=normal tissue from patient 5; T2=tumor
[a] Comparative crossing point
[b] No DNA added to reaction (negative control)

using alkaline phosphatase-conjugated anti-digoxigenin antibody and CDP-star substrate (Roche Diagnostics). Digital capture of light emission was carried out using Alpha Innotech chemiluminescent imaging instrument.

Standard curves for cycle number *vs.* log concentration for control (β-globin) and "unique" PYPAF5 gene using LC™ were established. They employed 11 concentrations of cDNA for both genes that used the same master mix for all reactions (each using 20 μl). Results showed linearity (11), in agreement with work on the external standard curve semi-quantitative method that showed linearity of amplification of target molecules over eight orders of magnitude (20). Figure 2 is an example of semi-quantitative real-time analysis on PYPAF5 gene using various samples (a), and melt carve analysis in blood of CRC (b) demonstrating detection specificity.

We then undertook pilot studies with the LC™ to compare relative gene expression using the cross point (CP) method, by employing the following: (a) six of seven "unique" colon cancer gene primers (PYPAF5, H1F1, HXCP2, TAX1BP2, OR2I4P and OR2A7). These genes were dubbed so as they are only expressed in human adenocarcinoma of the colon as ascertained by sequencing colon adenocarcinoma expressed sequence tag (EST) libraries in NCI's Cancer Genome Anatomy Project (CGAP) database [http://cgap.nci.gov] (1); (b) the GCC gene because it is a gene specific for colon cancer metastasis (21), and the CEA gene, which is currently the prognostic marker for recurrence in serum samples (9) on cells from normal and stages 3 or 4 of colon cancer LCM-enriched adenocarcinoma samples, colonocytes shed in stool and carcinoma cells from blood. Results are shown in Table I. It is apparent that for the β-globin standard there is no difference in expression between normal and colon cancer tissue (a CP value of ~32 was obtained in both cases). For the "unique" genes, it is apparent that there is differential expression between normal and cancer tissue, with very little or no amplification in normal tissue. For example for PYPAF5 and H1F1 genes, normal tissue obtained from patients 2 and 5 (N2 and N5) showed little or no

Table II. *ANOVA models for CP differences from β-globin.*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
CEA					
Group	2	158.84	79.42	43.42	0.0003
Residuals	6	10.97	1.83		
GCC					
Group	2	149.16	74.58	21.89	0.0018
Residuals	6	20.44	3.41		
H1F1					
Group	2	161.79	80.90	21.74	0.0018
Residuals	6	22.33	3.72		
HXCP2					
Group	2	83.30	41.65	5.69	0.0411
Residuals	6	43.90	7.32		
OR2A7					
Group	2	168.67	84.34	14.66	0.0049
Residuals	6	34.51	5.75		
OR2I4P					
Group	2	170.38	85.19	25.17	0.0012
Residuals	6	20.31	3.38		
PYPAF5					
Group	2	214.97	107.49	24.23	0.0013
Residuals	6	26.62	4.44		
TAX1BP2					
Group	2	212.80	106.40	22.93	0.0015
Residuals	6	27.84	4.64		

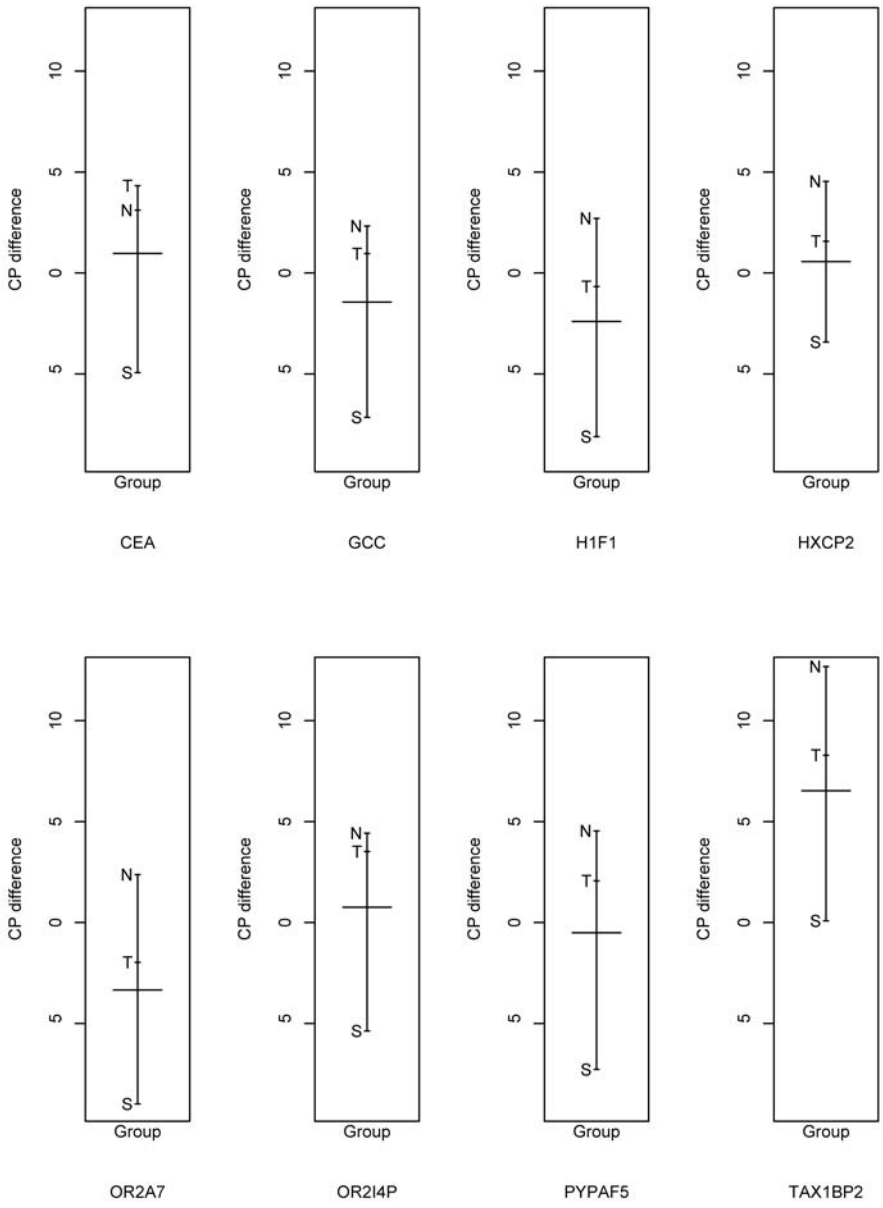

Figure 3. *Mean difference in CP level from β-globin for the three groups: tumor tissue (t), normal tissue (N), and blood or stool samples (S). The long hash mark is the mean of the three groups combined.*

amplification [average CP values of >36 for PYPAF5 gene, and ~34 for the HIF1 gene, respectively], which is practically similar to control without DNA present (referred to in Table I as Neg), while a greater amplification was detected much earlier (lower CP values) in tissue of colon cancer patients T2, T3, T4 and T5 (an average CP value of ~33 for PYPAF5 gene and 30.6 for H1F1 gene, respectively).

Results for the GCC gene were similar to the unique genes, whereas results for the CEA gene (an average of 34.56 for normal and > 36 for tumor tissue) in this study did not indicate early amplification in tumor tissue as shown by a low CP value for normal compared to tumorigenic tissue, which may suggest that this gene is not an ideal gene to use as a marker for recurrence. Amplification of all six "unique" genes, the GCC and CEA in stool and blood occurred early, although the quality and quantity of cDNA and reaction conditions were the same in tissue and exfoliated/minimally-invasive samples.

Statistical analysis on data employing ANOVA revealed that all eight ANOVA models, summarized in Table II,

were statistically significant (p-values < 0.05). The mean difference in CPs from β-globin for each of the three groups and each of the eight genes is presented in Figure 3. It must be noted that the sample sizes were very small, making it difficult to check on the normality assumption and the assumption of constant variance. Furthermore, the observations may not be independent since some values were taken on the same individual.

Discussion

Our experience working with RNA dictated that, to prevent its degradation, stool or dissected tissue samples were not allowed to stay unprocessed after removal from their normal environment for more than 20 min; freezing and thawing of stool samples for subsequent RNA extraction resulted in rupture of the colonocytes due to ice crystal formation, leading to degradation of RNA and failed PCR amplification because of exposure of the RNA to the hostile stool environment (5, 8). Hence, all RNA stool extractions used freshly collected stool, and extracted RNA was stably stored at -70˚C until further processing. Blood was also processed within 30 min of collection in $LiCl_2$ vacutainers, while sitting on ice (11). Commercial kits, neither cumbersome, nor necessitating specialized equipment, were employed to extract stable total RNA from all samples: cells in culture, tissue and exfoliated (8, 11).

Although the "perfect" standard gene does not exist, the choice of the reference gene utilized to normalize the expression of the target gene of interest is critical for the interpretation of results (22). Furthermore, the efficiencies of amplification of the control and experimental genes must be equal, as judged by similar slopes (8); in this study, the β-globin gene met this criterion.

Real-time amplification allows for a broad dynamic range of target molecule determination, and provides a means to precisely determine the product during the log/linear amplification phase, making the method one of the most sensitive techniques to quantitate low copy number transcripts (16). Although semi-quantitative PCR methods were thought to be inferior to quantitative competitive ones, side-by-side comparisons showed that both assays produced equivalent measures of template abundance (22), because methods employing real-time measure amplification in the logarithmic phase, whereas quantitative competitive measurements determine amplification during the linear phase (16). Roche's LC™ performed consistently and accurately, making it particularly suitable for quantitative work, as it is the only thermocycler on the market that uses air technology for instantaneous heating/cooling (14).

Although our findings demonstrate promising results, additional studies at various stages of colon cancer development (from 0 to 4) are needed: (a) using several marker genes that are only expressed in colon cancer (*e.g.*, genes PYPAF5, H1F1, TAX1BP2, OR2I4P, OR2A7, OR214P and FIZZ1) and other colon cancer-specific genes indicative of metastasis or recurrence, (b) employing various concentrations of RNA (to guard against errors due to different cDNA synthesis reactions) derived from colonocytes in stool, carcinoma cells in blood, and cells obtained from tissue of normal subjects and cancer patients to validate these marker studies, (c) utilizing several carefully selected housekeeping gene standards to ascertain similar slopes (indicating similar efficiencies) between the target gene and reference genes, (d) correlating the findings with various clinicopathological parameters in different genders and races as clinical usefulness of markers varies with stage, ethnicity and anatomic location of CRC, and (e) considering any other molecular parameters studied (mutations in oncogenes or tumor suppressor genes, protein truncations, *etc.*). Results from these investigations will lead to a predictive gene expression index [a product of two or more genes divided by the expression of another gene, or a product of two or more genes], which is considered a more reliable indicator of tumorigenesis than the expression of a single gene, before definitive conclusions can be drawn as to whether gene expression derived from carefully selected markers in stool or blood is a sensitive/specific diagnostic screening or prognostic predictor, respectively, for colon cancer.

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