Discovery of genes from feces correlated with colorectal cancer progression

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Abstract. Colorectal cancer (CRC) is considered to develop slowly via a progressive accumulation of genetic mutations. Markers of CRC may serve to provide the basis for decision-making, and may assist in cancer prevention, detection and prognostic prediction. DNA and messenger (m)RNA molecules that are present in human feces faithfully represent CRC manifestations. In the present study, exogenous mouse cells verified the feasibility of total fecal RNA as a marker of CRC. Furthermore, five significant genes encoding solute carrier family 15, member 4 (SLC15A4), cluster of differentiation (CD)44, 3-oxoacid CoA-transferase 1 (OXCT1), placenta-specific 8 (PLAC8) and growth arrest-specific 2 (GAS2), which are differentially expressed in the feces of CRC patients, were verified in different CRC cell lines using quantitative polymerase chain reaction. The present study demonstrated that the mRNA level of SLC15A4 was increased in the majority of CRC cell lines evaluated (SW1116, LS123, Caco-2 and T84). An increased level of CD44 mRNA was only detected in an early-stage CRC cell line, SW1116, whereas OXCT1 was expressed at higher levels in the metastatic CRC cell line CC-M3. In addition, two genes, PLAC8 and GAS2, were highly expressed in the recurrent CRC cell line SW620. Genes identified in the feces of CRC patients differed according to their clinical characteristics, and this differential expression was also detected in the corresponding CRC cell lines. In conclusion, feces represent a good marker of CRC and can be interpreted through the appropriate CRC cell lines.

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Abbreviations: CRC, colorectal cancer; SLC15A4, solute carrier family 15, member 4; CD44, cluster of differentiation 44; OXCT1, 3-oxoacid CoA-transferase 1; PLAC8, placenta-specific 8; GAS2, growth arrest-specific 2; Cq, quantification cycle; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Key words: colorectal cancer, fecal RNA, solute carrier family 15, member 4, serine/threonine kinase 17b, cluster of differentiation 44, 3-oxoacid CoA-transferase 1, placenta-specific 8, growth arrest-specific 2

Introduction

Colorectal cancer (CRC) is considered to develop slowly via the progressive accumulation of genetic mutations (1,2). Genes that regulate cell growth and differentiation must be altered in cancerous cells in the process of tumorigenesis (3,4). Markers of CRC may provide the basis for decision-making regarding intensive chemotherapy or molecule-targeting drugs in CRC patients (5-7). Therefore, the identification of markers may assist in cancer prevention, detection and prognostic prediction (5,8,9), thereby increasing survival rates (10). Molecular markers (11) have their own clinical significance in CRC (12).

In CRC, both sigmoidoscopy and colonoscopy are considered to be the gold standards regarding detection rates. However, these clinical examinations have drawbacks in terms of their risk and inconvenience (13,14). Molecular markers of CRC present in the peripheral blood of patients, including carcinoembryonic antigen and carbohydrate antigen 19-9, have been discussed in numerous reports, despite exhibiting

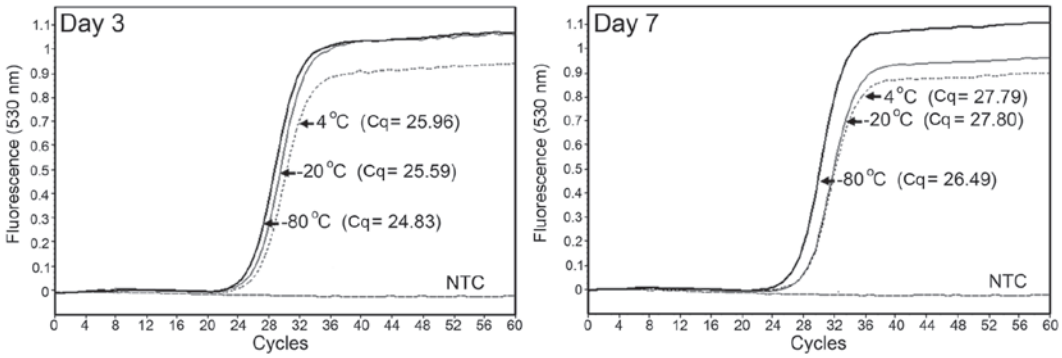

Figure 1. Quantitation of mouse β-actin in human feces. Each NIH 3T3-containing fecal sample was stored at 4, -20 and -80˚C for indicated periods. Day 3 equates to a 3-day storage, and day 7 equates to 7-day storage. Total RNA of NIH 3T3 cells in human feces was extracted and reverse transcribed into complementary DNA. The Cq value was used to indicate the expression level of the detected gene. NTC, non-template control; NIH, National Institutes of Health; Cq, quantification cycle.

poor specificity (15). In addition to the fecal occult blood test, the molecular detection of CRC using human feces has attracted attention in recent years (16-18). In fact, feces gather shedding cells from the colonic tract, including CRC cells, and respond to localized malignance (7,19,20). Not only DNA but also messenger (m)RNA molecules that are present in human feces faithfully represent CRC manifestations (17,21-24). For this reason, human feces are potentially appropriate material to gain an understanding of CRC development (25,26).

Gene expression is used for classifying tumors or predicting prognoses (27). The active genetic molecules that are differentially expressed in feces may be non-invasive candidates to indicate the pathogenic processes that underlie pharmacological responses. Studies of active genes in human feces have revealed specific molecular signatures of different CRC patients (28,29). Previously, several genes were reported as having differential expression in the feces of CRC patients (21,30). Furthermore, a number of these genes were correlated with cancer (20,21,24,31-34). The expression of the most significant of these genes must be characterized and explored in CRC cells (21,35,36).

To verify the clinical credibility of fecal molecules, the present study first assessed the stability of mRNAs from human fecal samples that were stored under different conditions. Subsequently, the most significant genes in CRC were verified using quantitative polymerase chain reaction (qPCR) in different CRC cell lines. The present results may shed light on the selection of the best treatment option for individual patients according to their significant fecal molecules.

Materials and methods

Quantitation of the mouse β-actin gene in human feces. To simulate the sloughed colonic cells present in human feces, $1x10^4$ mouse embryonic fibroblast cells [National Institutes of Health (NIH) 3T3 cells, gifted by Dr Shih-Ming Huang, National Defense Medical Center, Taipei, Taiwan] were added into 0.5 g of feces from a healthy volunteer (a 37-year-old male). The present study was approved by the Institutional Review Board of Cathay General Hospital (Taipei, Taiwan) as a research study. Each NIH 3T3-containing fecal sample was stored under different conditions (Fig. 1) in our specific buffer (30). The fecal total RNA was extracted and reverse transcribed into complementary (c)DNA as detailed in our previous reports (21,30). The mouse β-actin gene (NM_007393) was specifically quantified by qPCR on a LightCycler 1.5 instrument (Roche Diagnostics GmbH, Mannheim, Germany), according to the standard protocol (37). The primers and the TaqMan® probe used for quantifying the mRNA levels of mouse β-actin are listed in Table I. In addition, the quantification cycle (Cq) value was used to indicate the expression level of the detected gene (38).

Colonic cell lines and cell culture. In the present study, one normal colonic cell line [CCD-18Co, American Type Culture Collection (ATCC) CRL-1459], three early-stage CRC cell lines (SW1116, ATCC CCL-233; LS123, ATCC CCL-255; and SW480, ATCC CCL-228; ATCC, Manassas, VA, USA), and three late-stage CRC cell lines (SW620, ATCC CCL-227; Caco-2, ATCC HTB-37; and T84, ATCC CCL-248; ATCC) were used (39,40). In addition, one metastatic CRC cell line [CC-M3, Bioresource Colletion and Research Center (BCRC) 60450] was purchased from BCRC (Hsinchu, Taiwan) (41). With the exception of SW1116, SW480 and SW620, which were cultured in Leibovitz's L-15 Medium in a non-CO_2 incubator, other cells were cultured at 37˚C in a humidified 5% CO2 incubator with the medium recommended by ATCC, such as Eagle's minimum essential medium or Dulbecco's modified Eagle's medium. All culture medium contained fetal bovine serum to a final concentration of 10%.

Extraction of total cellular RNA and reverse transcription. Total cellular RNA was extracted from cultured cells using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Subsequently, 1 μg of total RNA was reverse transcribed into single-stranded cDNA using 0.5 μg of oligo(dT) primer and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Quantification of the mRNA levels of target genes in cells. The genes of interest were quantified in CCD-18Co cells or in the different CRC cell lines using the TaqMan® qPCR

Table I. Primers and TaqMan® probe for quantifying the messenger RNA levels of mouse β-actin.

Gene (symbol)	Accession no.	Primers (5'-3')	UPL no.
Mus musculus actin, beta (Actb)	NM_007393	F: AAGGCCAACCGTGAAAAGAT R: GTGGTACGACCAGAGGCATAC	#56

F, forward; R, reverse; UPL, Universal Probe Library.

Table II. Primers and TaqMan® probes for quantifying the messenger RNA levels of target genes.

Gene (symbol)	Accession no.	Primers (5'-3')	UPL no.
Solute carrier family 15, member 4 (SLC15A4)	NM_145648	F: GAGCAGTCACACAGACTTTGGT R: CAGGAGGGTAGCTCCTTGAA	#71
Cluster of differentiation 44 (CD44)	NM_001202555	F: CAAGCAGGAAGAAGGATGGAT R: AACCTGTGTTTGGATTTGCAG	#41
3-oxoacid CoA-transferase 1 (OXCT1)	NM_000436	F: ACTGGGTGTGATTTTGCAGTT R: GCAGCCTGGTACAAATATCCA	#84
Placenta-specific 8 (PLAC8)	NM_016619	F: CGTCGCAATGAGGACTCTCT R: CTCTTGATTTGGCAAAGAGTACAA	#56
Growth arrest-specific 2 (GAS2)	NM_005256	F: TGGGAGAAAAGATCCTCTTCATT R: TCAACAAATACCCTGCAAAAGTT	#75
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	NM_002046	F: CTCTGCTCCTCCTGTTCGAC R: ACGACCAAATCCGTTGACTC	#60

F, forward; R, reverse; UPL, Universal Probe Library.

Table III. Genes with potentially clinical significance in feces of CRC patients.

Comparison	Number of genes[a]	Representative gene	Used cell lines
Normal[b] vs. CRC	180	Solute carrier family 15, member 4	CCD-18Co, SW1116, LS123, Caco-2 and T84
Normal vs. AJCC stage I	167	Cluster of differentiation 44	CCD-18Co and SW1116
Non-metastasis vs. metastasis	9	3-oxoacid CoA-transferase 1	SW480 and CC-M3
Non-recurrence vs. recurrence	22	Placenta-specific 8 and growth arrest-specific 2	SW480 and SW620

[a]Genes with differential expression were identified as >2-fold (P<0.05). [b]Normal, healthy controls without any CRC symptoms. CRC, colorectal cancer; AJCC, American Joint Committee on Cancer.

approach, as aforementioned. The amplification primers and TaqMan® probes from the Universal ProbeLibrary Set, Human (Roche Diagnostics GmbH) used are listed in Table II. To avoid errors caused by sample-to-sample differences in RNA quantity, the normalization of each gene was performed using the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NM_002046). LightCycler 4.05 Software (Roche Diagnostics GmbH) was used to analyze the PCR kinetics.

Statistical analysis. Gene expressions of two groups were analyzed for significance using the Student's t-test. The calculations were made with SPSS software (v.16.0; SPSS, Inc. Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Feasibility of total fecal RNA as a marker of CRC and custom-made microarrays for CRC patients. As indicated in Fig. 1, three Cq values (25.96 at 4˚C, 25.59 at -20˚C and 24.83 at -80˚C) after a 3-day storage were not remarkably different to those detected at day 0 (25.61). Similar results were obtained after a 7-day storage; however, the difference was slightly larger. By applying this technique of fecal RNA purification, numerous genes that were expressed differentially

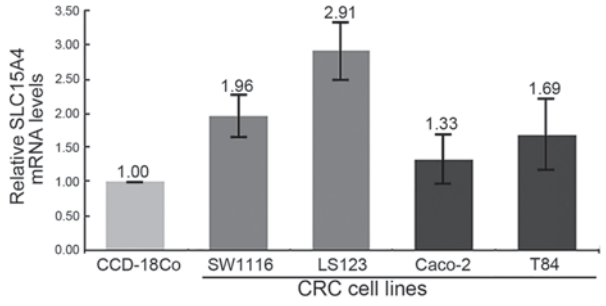

Figure 2. Relative mRNA levels of SLC15A4 in CRC cells. The expression of SLC15A4 (NM_145648) was quantified by quantitative polymerase chain reaction and normalized to the expression of endogenous glyceraldehyde 3-phosphate dehydrogenase (NM_002046). Normal colonic cell line, CCD-18Co; early-stage CRC cell lines, SW1116 (AJCC stage I) and LS123 (AJCC stage II); late-stage CRC cell lines, Caco-2 and T84 (AJCC stage IV). SLC15A4, solute carrier family 15, member 4; AJCC, American Joint Committee on Cancer; mRNA, messenger RNA; CRC, colorectal cancer.

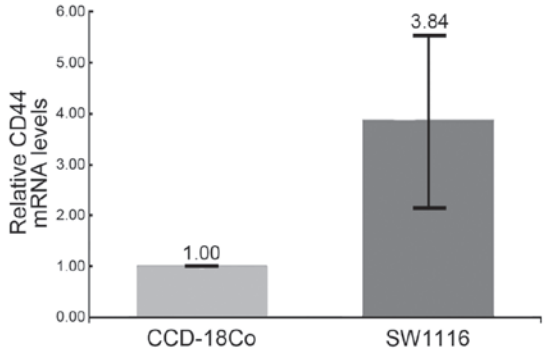

Figure 3. Relative mRNA levels of CD44 in early-stage CRC. The expression of CD44 (NM_001202555) was quantified by quantitative polymerase chain reaction and normalized to the expression of endogenous glyceraldehyde 3-phosphate dehydrogenase (NM_002046). Normal colonic cell line, CCD-18Co; early-stage CRC cell line, SW1116 (American Joint Committee on Cancer stage I). CD44, cluster of differentation 44; mRNA, messenger RNA; CRC, colorectal cancer.

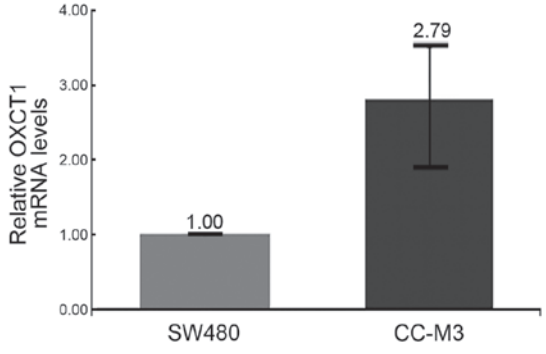

Figure 4. Relative mRNA levels of OXCT1 in metastatic CRC. The expression of OXCT1 (NM_000436) was quantified by quantitative polymerase chain reaction and normalized to the expression of endogenous glyceraldehyde 3-phosphate dehydrogenase (NM_002046). Non-metastatic CRC cell line, SW480; metastatic CRC cell line, CC-M3. mRNA, messenger RNA; CRC, colorectal cancer; OXCT1, 3-oxoacid CoA-transferase 1.

in the feces of CRC patients were identified, as assessed by analysis of whole-genome oligonucleotide microarrays (30). As summarized in Table III, genes with a significantly

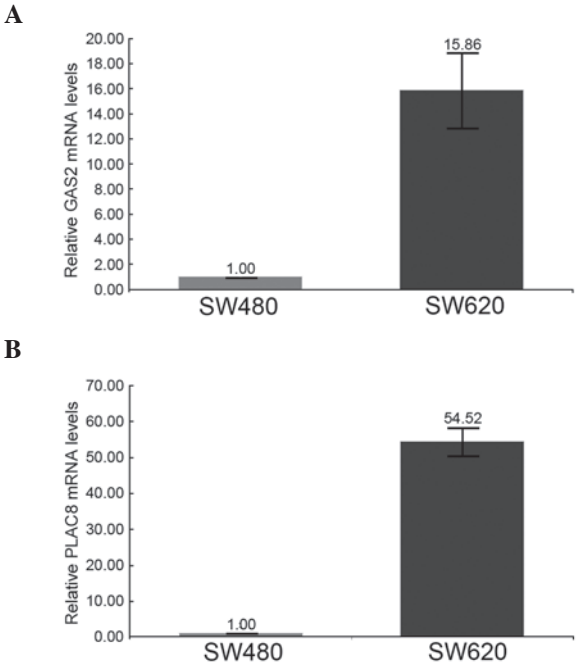

Figure 5. Relative mRNA levels of genes in recurrent CRC. (A) GAS2 (NM_005256) and (B) PLAC8 (NM_016619) were quantified by quantitative polymerase chain reaction and normalized to the expression of endogenous glyceraldehyde 3-phosphate dehydrogenase (NM_002046). Non-recurrent CRC cell line, SW480; recurrent CRC cell line, SW620. mRNA, messenger RNA; CRC, colorectal cancer; PLAC8, placenta-specific 8; GAS2, growth arrest-specific 2.

differential expression (>2-fold, P<0.05) were selected from the feces of a group of 16 subjects that consisted of 5 noncancerous individuals and 11 CRC patients at different American Joint Committee on Cancer (AJCC) stages (2 at AJCC stage I, 3 at stage II, 3 at stage III and 3 at stage IV).

Validation of genes that were differentially expressed in CRC cell lines. Partial genes that were highly expressed in the feces of CRC patients were verified using CRC cell lines (Figs. 2-5 and Table III). For example, solute carrier family 15, member 4 (SLC15A4, NM_145648) was upregulated in the majority of feces of CRC patients. The expression of SLC15A4 was also increased in four CRC cell lines (SW1116, LS123, Caco-2 and T84) at different AJCC stages compared with the normal colonic cell line CCD-18Co (Fig. 2).

To identify the initial stages of CRC, 167 genes that exhibited differential expression in early-stage CRC or in CRC patients without symptoms were selected. Among these 167 genes, a cell-surface molecule, cluster of differentiation (CD)44 (NM_001202555) was upregulated (42) in the SW1116 cell line (3.84-fold), which was previously diagnosed as AJCC stage I (Fig. 3). To date, the majority of the genetic research conducted on metastatic or recurrent CRC is confined to a single molecule (43-45). It is known that distant metastasis is the major cause of mortality in CRC patients (46). However, few studies have investigated the profiles of genetic variation in these CRC patients. Thus, fecal total RNA was used to distinguish metastatic or recurrent CRC patients from other CRC patients in the present study. The results revealed that one gene involved in extrahepatic ketone-body catabolism, 3-oxoacid CoA-transferase 1 (OXCT1,

NM_000436), was expressed at higher levels (2.79-fold) in the metastatic CRC cell line CC-M3 than in the non-metastatic cell line SW480 (Fig. 4). In addition, two genes, growth arrest-specific 2 (GAS2, NM_005256) and placenta-specific 8 (PLAC8, NM_016619), which are involved in recurrent CRC, were detected in the SW620 cell line, which is the lymph-node metastatic derivative of the SW480 cell line (47,48). As indicated in Fig. 5, both GAS2 (15.86-fold) and PLAC8 (54.52-fold) were upregulated in the SW620 cell line compared with the SW480 cell line.

Discussion

The differentiation of CRC patients from non-cancer individuals or CRC patients with different clinical characteristics is crucial in CRC treatment. However, the current staging system used for CRC, which is based on the tumor-node-metastasis classification, does not yield a reliable personalized prediction of prognosis (49). This can be improved by employing molecular parameters in addition to the staging system (50).

In recent years, human feces have been used as research material in CRC (51). Both fecal DNA and RNA are known to represent CRC-related molecular targets (51-53). Our previous studies also reported various molecules that are differentially expressed in human feces (30). Significant gene profiles were acquired computationally by comparing different group settings according to clinical characteristics. In the present study, the feasibility of total fecal RNA as a marker of CRC was first verified using exogenous mouse cells. Subsequently, different CRC cell lines were used to validate the differentially expressed genes in feces. The significant molecules detected in CRC cell lines may provide novel insights into colorectal carcinogenesis and personalized prediction in a non-invasive manner using human feces.

For example, upregulation of SLC15A4 was detected in the feces of CRC patients and in CRC cell lines at different stages (AJCC stages I-IV). Expression of SLC15A4, a histidine transporter, was previously observed in the gastrointestinal tract (54). This histidine transporter coordinates mechanistic target of rapamycin-dependent inflammatory responses and may promote colitis (55,56). In the present study, two early-stage CRC cell lines, SW1116 and LS123, exhibited a higher expression level of SLC15A4 than normal colonic cells and late-stage CRC cell lines. Furthermore, an anti-inflammatory function may contribute to antitumor activity (57). Thus, SLC15A4 may participate in the initial inflammation-induced colorectal dysplasia. This result may be associated with another marker, CD44, which was detected in the feces of CRC patients at AJCC stage I. CD44 is of functional importance for tumor initiation and progression in CRC (58). In another animal study, downregulated CD44 was able to reduce tumor growth significantly (59). Recently, CD44 was further proposed to contribute to targeted therapeutic strategies due to its role in sensing the extracellular environment (60). These findings are in agreement with the results obtained in the present study for the fecal samples of early-stage cancer groups and for SW1116 cells. Taken together, our findings revealed that the detection of SLC15A4 and CD44 in feces may aid to identify the initial CRC cells. Another gene that was identified in the feces of CRC patients at AJCC stage IV was OXCT1 (61-63). Increased expression of OXCT1 has been observed in numerous human cancers. As detected in the present study in metastatic CRC, a substantially elevated level of expression of OXCT1 has been reported in association with metastatic cancers (62,63), which suggests that OXCT1 may be a potential marker of late-stage CRC and can be detected in feces.

In fact, the two genes involved in CRC recurrence described in the current study were also reported in other human cancers. For example, GAS2 was expressed at a high level in chronic myeloid leukemia cells, and the inhibition of GAS2 impaired tumor growth. PLAC8 was also upregulated in other human leukemic cells and induced apoptosis resistance (64). In addition, PLAC8 overexpression was further linked to intestinal stem cells in CRC (65). Our current results appear to agree with these reports due to the high expression levels of GAS2 and PLAC8 detected in the feces of relapsed CRC patients and in the recurrent CRC cell line SW620.

Genes involved in CRC tumorigenesis or with uncharacterized functions may be potential markers that could aid in CRC detection, diagnosis, treatment or prognostic prediction (66). However, upregulated genes were frequently observed during the process of CRC tumorigenesis (67). In the present study, CRC cell lines were used to validate the genes that were significantly upregulated in the feces of CRC patients. Our results suggest that CRC cell lines can respond to differential gene expression in feces. Thus, the present study focused on detecting fecal RNA in association with tumor initiation, recurrence and liver metastasis in CRC. Clinically, pathological factors, alone or in combination, cannot perfectly identify CRC patients or make a personalized prediction of recurrence (49,68). The molecules involved in CRC pathogenesis may act as markers of early CRC diagnosis or may be used to stratify susceptible patients into appropriate screening or surveillance programs (69). In other words, the genetic understanding of CRC has led to the introduction of molecular proposals that exemplify the knowledge translated from basic science to clinical care (10). The possible clinical application of non-invasive molecules provides a useful platform in molecular medicine and translational research. Genes expressed in the feces of CRC patients varied in the present study according to the clinical characteristics of the individuals, and these differential expression levels also arose in the corresponding CRC cell lines. In conclusion, feces represent a good marker of CRC and can be interpreted using the appropriate CRC cell lines.

Acknowledgements

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