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DNA methylation biomarker candidates for early detection of colon cancer

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Abstract

Promoter CpG island hypermethylation of tumor suppressor genes is a common hallmark of all human cancers. Many researchers have been looking for potential epigenetic therapeutic targets in cancer using gene expression profiling with DNA microarray approaches. Our recent genome-wide platform of CpG island hypermethylation and gene expression in colorectal cancer (CRC)

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cell lines revealed that *FBN2* and *TCERGIL* gene silencing is associated with DNA hypermethylation of a CpG island in the promoter region. In this study, promoter DNA hypermethylation of *FBN2* and *TCERGIL* in CRC occurs as an early and cancer-specific event in colorectal cancer. Both genes showed high frequency of methylation in colon cancer cell lines (>80% for both of genes), adenomas (77% for *FBN2*, 90% for *TCERGIL*, $n=39$), and carcinomas (86% for *FBN2*, 99% for *TCERGIL*, $n=124$). Bisulfite sequencing confirmed cancer-specific methylation of *FBN2* and *TCERGIL* of promoters in colon cancer cell line and cancers but not in normal colon. Methylation of *FBN2* and *TCERGIL* is accompanied by downregulation in cell lines and in primary tumors as described in the Oncomine™ website. Together, our results suggest that gene silencing of *FBN2* and *TCERGIL* is associated with promoter DNA hypermethylation in CRC tumors and may be excellent biomarkers for the early detection of CRC.

Keywords

DNA hypermethylation; Biomarker; Early detection; Colorectal Cancer (CRC); *FBN2*; *TCERGIL*

Introduction

Colorectal cancer (CRC) remains the second leading cause of cancer-related mortality in the USA [1]. Current screening modalities have resulted in only a modest decrease in mortality and have failed to achieve high public participation (<50%) [2, 3]. Strategies such as colonoscopy are invasive, whereas stool occult blood tests are of limited use because of the need for repeated measurements and interference by dietary components. The use of biomarkers, specifically epigenetic biomarkers, in serum, plasma, and stool are promising to close that gap between invasive but dependable tests and noninvasive but unreliable studies.

The past few years have seen an explosion of interest in the epigenetics of cancer. Epigenetic alterations have been widely recognized to play an important role in the development of cancer. Aberrant hypermethylation in the promoter regions of the tumor suppressor genes at CpG islands has been recognized as one of the hallmarks of cancer [4]. This hypermethylation of gene promoters is associated with downregulation of expression and the inactivation of critical tumor suppressor genes in human cancers [5]. Methylation of multiple genes in colorectal (CRC) cancer has been described including *hMLH1*, *p16^{INK4A}*, *APC*, *MGMT*, *sFRP1*, *GATA-4* and *GATA-5* [4, 6, 7]. More recently, DNA hypermethylation of *TFPI2* and *SOX17* genes have been detected in early stage of CRC patients [8].

We previously described a genome-wide expression array-based approach to identify the “DNA hypermethylome” in colon cancer estimated to contain about 500 hypermethylated genes per individual tumor [9]. Using this platform, we have identified a number of genes that are hypermethylated and underexpressed in colon cancers but not in normal tissues [9]. Furthermore, DNA methylation changes in cancer represent an attractive therapeutic target since epigenetic alterations are more readily reversible than genetic events [10]. However, the great strength of DNA methylation in the clinic promises to be in the area of molecular diagnostics and early detection.

Fibrillin 2 (*FBN2*), is an extracellular matrix protein. It is associated with elastic fibers in several tissues and is believed to serve as a ligand for α v β 3 integrin, the latter being a known morphogen. *FBN2* was first discovered to be expressed in the mesenchymal tissues and at the epitheliomesenchymal interface. It has also been demonstrated that the *FBN2* antisense oligodeoxynucleotide can induce dysmorphogenesis of the lung explants, suggesting that *FBN2* plays a key role in lung development [11]. Recently, the loss of *FBN2*

expression due to promoter methylation was identified in pancreatic cancer cell lines by means of high-throughput microarray analysis [12].

Transcription elongation regulator 1-like (*TCERG1L*) gene located in chromosome 10, emerged as showing frequent cancer-specific methylation in our microarray-based approaches [9]. *TCERG1L* gene may have similar biological role with *TCERG1* (transcription elongation regulator 1), located on human chromosome 5q32. *TCERG1* (also known as TAF2S; TATA box-binding protein-associated factor 2S, CA150; transcription factor CA150) was first described as a transcriptional elongation regulator found in human immunodeficiency virus type 1 (HIV-1), tat-responsive HeLa nuclear fractions [13]. *TCERG1L* may have a function in the elongation-related factors in HeLa nuclear extracts [14] even though little else is known so far. Prior to this study, *TCERG1L* has not been described as being involved in cancer.

Here, we investigated promoter DNA methylation of *FBN2* and *TCERG1L* genes in CRC cell lines and primary tumors. We present data indicating that *FBN2* and *TCERG1L* genes are hypermethylated in most of CRC cell lines and primary tumors in a cancer-specific manner. Additionally, we demonstrate that methylation of *FBN2* and *TCERG1L* occurs in precancerous colon polyps suggesting that methylation of both of these genes is an early event in CRC. We suggest that *FBN2* and *TCERG1L* methylation could be a useful biomarker for noninvasive detection of precancerous and cancerous colon and rectal tumors.

Materials and methods

Cell culture and treatment

Colorectal cell lines (HCT116, RKO, HT29, SW480, DLD1, COLO 320, SW48, Lovo, Caco-2, and SW620) were obtained from ATCC (Manassas, VA, USA) and cultured in appropriate medium and under conditions described by ATCC, with media obtained from Invitrogen (Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). DKO cells (HCT116 cells with genetic disruption of *DNMT1* and *DNMT3b*) were cultured as previously described [15]. For demethylation studies, cultured cells were treated with 1 μ M 5-aza-2'-deoxycytidine (DAC; Sigma, St. Louis, MO, USA) for 72 h with media changed every 24 h. Trichostatin A (TSA; Sigma, St. Louis, MO, USA) was obtained from Sigma and used to treat cells at concentration of 300 nM for 18 h. Mock drug treatments were performed in parallel with drug-free PBS.

Primer design

For expression studies using RT-PCR, we designed primers using the open access program Primer3 (<http://frodo.wi.mit.edu/primer3>). Primer sequences for methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing analysis were designed using MSPPrimer (<http://www.mspprimer.org>) and their location in the *FBN2* and *TCERG1L* promoter is indicated in Fig. 3. All primer sequences are listed on Table 1.

Gene expression and methylation analyses

For MSP analysis, DNA was extracted following a standard phenol–chloroform extraction method. Bisulfite modification of genomic DNA was carried out using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). We performed methylation analysis of the *FBN2* and *TCERG1L* promoter using MSP primer pairs covering the putative transcriptional start site in the 5' CpG island with 1 μ l of bisulfite-treated DNA as template and JumpStart Red Taq DNA Polymerase (Sigma, St. Louis, MO, USA) for amplification as previously described [16].

Quantitative RT-PCR

Total RNA was extracted from all cell lines using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), and was treated using the DNaseI protocol (QIAGEN, Valencia, CA, USA). One microgram total RNA was subjected to the Superscript II first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. For quantitative real-time analyses, the iQTM SYBR Green Supermix kit (Bio-Rad) was used and the amplification conditions consisted of an initial 10-min denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension for 30 and 60 s, respectively. A Bio-Rad CFX384 real-time PCR System was used (Bio-Rad) and we used the comparative Ct method to compute relative expression values. We averaged expression of GAPDH as internal reference genes to normalized input cDNA. Also, we used normal colon and DKO cells (HCT116 cells with genetic disruption of *DNMT1* and *DNMT3b*) cDNA samples as positive controls for *FBN2* and *TCERG1L* gene expression.

Bisulfite sequencing analysis

One microgram of genomic DNA from each sample was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) following manufacturer's protocol. MSP primer sequences are provided in Table 1. The PCR amplicons were gel-purified and subcloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). At least ten clones were randomly selected and sequenced on an ABI3730xl DNA analyzer to ascertain the methylation patterns of each locus.

Primary human tissue samples

Primary tissue samples were obtained from the archives of the Department of Pathology, Johns Hopkins University with Institutional Review Board approval and Health Insurance Portability and Accountability Act compliance. We compiled data from 124 formalin-fixed and paraffin-embedded primary CRCs of tumor stages I to IV (stage I—*n*=33, stage II—*n*=37, stage III—*n*=36, stage IV—*n*=18; mean age, 65.2 years). We also examined 39 colorectal adenomas from patients without an associated invasive CRC (tubular—*n*=17, villous—*n*=22). We compared these samples to 20 normal colon controls of patients without any colorectal neoplasm (Table 2).

Results

Correlation between DNA promoter methylation and gene silencing of *FBN2* and *TCERG1L* in colon cancer cell lines

We previously developed an expression microarray technique to characterize the spectrum of hypermethylated genes in cancers [9]. Based on our previous study, some of the cancer-specific methylated genes detected in early stage of colon cancer could also function as diagnostic and prognostic biomarkers [8, 9]. *FBN2* and *TCERG1L* genes were identified in our hypermethylome approach and both have a dense CpG island in the promoter region upstream from the transcriptional start site (Fig. 3). To query these genes for methylation, we designed primers in promoter region of each gene for methylation-specific PCR (MSP) assays. Having confirmed our MSP primers and their monitoring of methylation density, we then tested both primers in ten different colon cancer cell lines (Fig. 1). We found complete methylation of *FBN2* promoter region in all ten colon cancer cell lines (100%, 10/10) (cell lines; HCT116, SW480, RKO, HT29, Caco-2, Lovo, COLO 320, COLO 205, DLD1, SW48, and SW620) (Fig. 1a). *TCERG1L* also showed complete methylation (80%, 8/10) in the majority of colon cancer cell lines except for Colo320 and Lovo (Fig. 1b). To ensure that methylation in these samples was associated with downregulation of gene expression, we then tested the expression of *FBN2* and *TCERG1L* using RT-PCR in a same panel of CRC

cell lines with/without DAC and TSA treatment. Quantitative real-time PCR results confirmed re-expression of *FBN2* and *TCERG1L* genes after treatment with DAC, a demethylating agent, in most of CRC cell lines. Furthermore, we detected little or no expression of *FBN2* and *TCERG1L* in ten CRC lines, but all of these lines showed significantly increased expression of *FBN2* and *TCERG1L* following DAC treatment (Fig. 1a,b). In the case of *TCERG1L*, even though it has basal expression in Lovo cells, its expression level increased after DAC treatment compared to basal expression. Interestingly, expression levels by RT-PCR of these two genes are not increased after treatment with TSA, a histone deacetylase inhibitor. Therefore, inactivation of *FBN2* and *TCERG1L* gene appears to be primarily regulated by promoter DNA hypermethylation in colon cancers.

Aberrant hypermethylation of the *FBN2* and *TCERG1L* gene in primary CRC tumors

Next, we expanded this study in a large cohort of CRC patient samples. One hundred twenty-four CRC samples (stages I–IV), 39 adenomas, and 20 normal patient samples were examined. Neither gene displayed methylation in normal colon samples (Fig. 2a). *FBN2* was found to be methylated in 90% (30/33) of stage I, 83% (31/37) of stage II, 91% (33/36) of stage III, and 72% (13/18) of stage IV colon cancers. *TCERG1L* was found to be methylated in 100% (33/33) of stage I, 100% (37/37) of stage II, 97% (35/36) of stage 3, and 100% (18/18) of stage 4, CRC samples (Fig. 2b). This data indicates that *FBN2* and *TCERG1L* gene methylation is cancer-specific. Moreover, we tested *FBN2* and *TCERG1L* promoter methylation in tubular and villous adenomas which are considered precancerous. Interestingly, *FBN2* methylation was observed in 71% of tubular adenomas (12 of 17), and 82% of villous adenomas (18 of 22). *TCERG1L* methylation was observed in 88% of tubular adenomas (15 of 17), and 91% of villous adenomas (20 of 22). This data strongly suggest that *FBN2* and *TCERG1L* hypermethylation occur as very early event during the progression of primary CRC. Therefore, detecting of DNA methylation for *FBN2* and *TCERG1L* genes could be useful as early detection biomarker in CRC.

We also confirmed DNA methylation status of *FBN2* and *TCERG1L* genes in a representative colon cancer cell line (HCT116) and primary CRC tumor sample by bisulfite sequencing analysis. Bisulfite sequencing of *FBN2* and *TCERG1L* gene promoter region showed dense DNA methylation in the colon cancer cell line HCT116 and CRC tumor sample and no methylation was detected in DKO cells and normal colonic tissues (Fig. 3). This data further supports our premise that *FBN2* and *TCERG1L* are methylated in a cancer-specific fashion in CRC.

FBN2 and *TCERG1L* gene expression is downregulated in CRC

Aberrant DNA hypermethylation has been well known to be associated with gene silencing in cancer. We observed *FBN2* and *TCERG1L* gene DNA hypermethylation is correlated with decreased gene expression and reactivated by DAC compare to basal expression level in colon cancer cell lines (Fig. 1a, b), suggesting that both of gene expression in colon cancer cell lines where methylation tightly correlated with lack of expression. However, since we have seen DNA hypermethylation of both genes in most of colon primary tissues, we wonder if these expression levels are correlated with DNA methylation status in colon primary tumors. Therefore, we utilized the gene expression microarray data previously published on Oncomine™ (Compendia Bioscience, Ann Arbor, MI, USA). The Oncomine™ database is a web-based data-mining platform aimed at facilitating gene discovery from genome-wide expression analyses in cancer [17]. We therefore queried the expression of *FBN2* and *TCERG1L* in colon primary tumors from several studies (Table 3). Very interestingly, we found that both genes are significantly downregulated in six independent colon primary tumor datasets [18–21]. This information, in combination with

our cell lines data, strongly suggests that *FBN2* and *TCERGIL* are methylated and downregulated in a cancer-specific fashion in CRC.

Discussion

Genome-wide approaches have contributed to our understanding of the molecular pathways driving tumorigenesis as well as providing useful new biomarkers for cancer risk assessment, early diagnosis, and prognosis [22]. Previous studies have confirmed that our microarray strategy is an effective approach to identify genes that are silenced by promoter hypermethylation in colon and breast cancer [9, 23, 24]. DNA methylation of gene promoters is among the earliest and most frequent oncologic alterations. A number of genes are commonly hypermethylated in CRC, however, genes methylated in early-stage tissues with high frequency are rare [9, 24, 25]. Previously, we have reported aberrant methylation of *TFPI2* and *SOX17* in CRC using our established array-based platform [26]. *TFPI2* gene promoter methylation has been identified in early stage of colon cancer and is highly sensitive in stool samples making it an excellent choice for noninvasive surveillance for CRC [8].

We have now discovered two additional genes which show cancer-specific methylation which are detected with high frequency in early stages of colorectal cancer formation. *FBN2* and *TCERGIL* are genes harboring cancer-specific promoter methylation in human colorectal cancer. Promoter DNA hypermethylation of *FBN2* and *TCERGIL* in CRC (86% for *FBN2*, 99% for *TCERGIL*, $n=124$, respectively) are frequent and cancer-specific events. Hypermethylation of *FBN2* has also been reported in pancreatic [12] and lung cancers [27–29], yet has not been described in CRC. *TCERGIL* has not previously been described in cancer. To our knowledge, this is the first description of *FBN2* and *TCERGIL* promoter methylation occurring as an early event in colon cancer progression.

Methylation biomarkers for the detection of an oncologic process requires that the DNA be obtained in a noninvasive fashion, either through blood, stool, or mucous. *TFPI2* has been shown to be sensitive and specific in stool samples. Plasma and serum biomarkers have also been shown to have relatively high specificity in CRC [30–32]. Therefore, testing our genes in stool, plasma, or serum, samples is necessary to confirm whether either gene could be useful biomarker for early detection in cancer in the near future. Additionally, other excellent early detection biomarkers have already been published by us and others [8, 33]. Any future study would need to test *FBN2* and *TCERGIL* in combination with *TFPI2* in colon cancer. It has been well known that methylation of CpG islands in gene promoter regions is associated with aberrant silencing of transcription and is a mechanism for inactivation of tumor suppressor genes (p16, APC, Rb) from many other studies [4, 25]. Recently, newly identified hypermethylated genes in cancer by genome-wide microarray approach showed its biological roles such as tumor-suppressive effect in cancer cells [8, 34]. Therefore, it could have a possibility that *FBN2* and *TCERGIL* might have tumor-suppressive effect in colon cancer but further study is need to define the functions of *FBN2* and *TCERGIL* in colon tumorigenesis.

In conclusion, *FBN2* and *TCERGIL* gene methylation is an early and frequent event in precancerous and cancerous lesions of the colon and rectum. Furthermore, this methylation is cancer-specific and was not detected in any normal colon samples. Finally, the methylation of *FBN2* and *TCERGIL* is associated with a phenotypic underexpression these gene products. These three factors warrant further examination of *FBN2* and *TCERGIL* in noninvasive studies for the early detection of colorectal cancer. We have reported for the first time that *FBN2* and *TCERGIL* promoter methylation is a feasible epigenetic marker for early detection of CRC and may be useful for CRC screening in the future.

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Fig. 1.

Epigenetic inactivation of *FBN2* and *TCERG1L* in colon cancer cell lines. Methylation analysis of (a) *FBN2* and (b) *TCERG1L* gene promoter (Upper panel) and real-time RT-PCR results for (c) *FBN2* and (d) *TCERG1L* gene in colon cancer cell lines after DAC and TSA treatment with mock in each cell lines. *GAPDH* was used as control for equal amplification. PCR products recognize unmethylated (U) and methylated (M). DKO cells [15] were used for unmethylated control. IVD = *in-vitro* methylated control, ddH₂O=water control containing no DNA. M, A, and T indicate mock, DAC treated cells, Trichostatin A (TSA) treated cells, respectively

Fig. 2.

Epigenetic inactivation of *FBN2* and *TCERG1L* in colon primary tissues versus normal colon tissues. (a) normal colon tissues (b) primary CRC tissues. M = methylation signal; U = unmethylated signal. IVD = in vitro methylated DNA. ddH₂O=water control adding no DNA. Numbers represent normal colon (NA1-20). NC indicates normal colon. Bar graph showing MSP results of all primary samples (stage I-IV: n=124) and adenomas (tubular adenoma (TA), villous adenoma (VA), n=39)

(A) *FBN2*

(B) *TCERG1L* **Fig. 3.**

Representative Bisulfite sequencing results of *FBN2* (a) and *TCERG1L* (b) in HCT116, DKO, a primary CRC (CRC20), and normal colon (NC10). Open and filled circles represent unmethylated and methylated CpG sites, respectively, and each row represents a single clone. Location of CpG sites (*FBN2*: upstream region from - 475 to -64; *TCERG1L*: upstream region from -556 to -275) relative to the transcription start sites (TIS) of Exon 1 is shown Fig. 3. Both genes bisulfate sequencing region include MSP analysis amplicons

Table 1

Primers for MSP, RT-PCR, and bisulfite sequencing analysis

Gene		S (5'-3')	AS(5'-3')
<i>FBN2</i>	Meth	GGGTTTTTAAAATTTTCGCGTCGC	CTACGAAACCGAACGAAAATACG
	Unmeth	GTTTTGTGGGTTTTTAAAATTTTGTGTGTG	AAATAACAACCTACAAAACCAACAAAATACA
	RT	AGGGATCCTTCATTTGCCAC	GCCCGGGGTATTTACACACT
	BST	CTTCCAACCCYACCTTC	GTTTTTAGAAGAAGAGGAGGG
<i>TCERGIL</i>	Meth	GGTCGTTTGCCTCGGATTTC	CTACCCAACGCGAACTAAAAACG
	Unmeth	TTTGGGGTTGTTTGTGTGGATTG	CATATCCCACTACCCAACACAAACTAAAAACA
	RT	TGCTCTTGCAACCCCTATTG	TGAGGTGCTAACACCACCGT
	BST	AATTTGTTTGGTTTATTGTGTAATAGAAAT	CTAATAACCTCTAACCCTCTAA

Table 2

Baseline characteristics of CRC patients in this study

Characteristics	N (%)
CRC (<i>n</i> =124)	
Age (years)	
Median	65.2
Sex	
Male	68 (55%)
Female	56 (45%)
Race	
White	84 (68%)
Black	33 (27%)
Asian	2 (1%)
Other	5 (4%)
Grade	
Well-differentiated	39 (31%)
Moderately differentiated	63 (51%)
Poorly differentiated	22 (18%)
Location	
Right	71 (58%)
Left	51 (41%)
Right and left	2 (1%)
Stage	
I	33 (27%)
II	37 (30%)
III	36 (29%)
IV	18 (14%)
Adenomas (<i>n</i> =39)	
Tubular	17 (44%)
Villous	22 (56%)
Normal colon (<i>n</i> =20)	20

Table 3*FBN2* and *TCERG1L* gene expression changes in colon tumor samples from ONCOMINE database

Colon cancer	Sample no.	<u>Median log2 ratio^a</u>		References
		<i>TCERGIL</i>	<i>FBN2</i>	
Adenomas				
Colon villous adenoma	17	−1.862	−1.078	Not published since 2005 (Bittner Group)
Carcinoma				
Colon adenocarcinoma	41	−1.41	−1.142	Kaiser et al. [18]
Colon mucinous adenocarcinoma	13	−1.454	−1.184	Kaiser et al. [18]
Colon adenocarcinoma	137	−0.698	−0.51	Jorissen et al. [19]
Colorectal adenocarcinoma	177	−1.465	−1.229	Smith et al. [20]
Colon adenocarcinoma	239	−1.121	−0.808	Not published since 2005 (Bittner Group)
Colon mucinous adenocarcinoma	42	−1.623	−0.794	Not published since 2005(Bittner Group)
Colorectal carcinoma	84	−1.459	−1.598	Watanabe et al. [21]

^alog₂ median-centered intensity which is array-based gene expression log-fold changes. Oncomine threshold by *p* value (<0.0001), fold change (>2)