BASIC–ALIMENTARY TRACT

A Genome-Wide Search Identifies Epigenetic Silencing of *Somatostatin*, *Tachykinin-1*, and 5 Other Genes in Colon Cancer

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Background & Aims: Gene silencing via promoter hypermethylation is a central event in the pathogenesis of cancers. To identify novel methylation targets in colon cancer, we conducted a genome-wide, microarray-based, in silico, and epigenetic search. **Methods:** Complementary DNA microarray experiments were first performed to identify genes down-regulated in primary colon cancers and up-regulated in colon cancer cell lines after global DNA demethylation by 5-aza-2′-deoxycitidine. Candidate methylation targets were then identified by combining these microarray data with *in silico* genetic and functional searches. Candidate genes recognized by these searches were further investigated for promoter hypermethylation in colon cancer using methylation-specific polymerase chain reaction. **Results:** We identified 51 novel and 3 known candidate methylation targets. Subsequent epigenetic analysis revealed that primary colon cancers demonstrated frequent methylation of *somatostatin* (*SST*, 30 of 34 cases, 88%) and the substance P precursor gene *tachykinin-1* (*TAC1*; 16 of 34 cases, 47%). *TAC1* methylation intensity was significantly higher in Dukes A/B than in Dukes C/D cancers ($P = .01$). *SST* methylation intensity was significantly higher in low-level microsatellite instability (MSI-L) than in non-MSI-L cancers ($P = .02$). Methylation was associated with messenger RNA down-regulation for both *SST* and *TAC1*. Furthermore, we isolated 5 additional novel promoter methylation targets: *NELL1*, *AKAP12*, *caveolin-1*, *endoglin*, and *MAL*. **Conclusions:** These data strongly suggest that *SST* and *TAC1* are involved in colon carcinogenesis. Further studies are now indicated to elucidate mechanisms underlying their involvement in colon cancer and their values as clinical biomarkers. *NELL1*, *AKAP12*, *caveolin-1*, *endoglin*, and *MAL* are also promising tumor suppressor gene candidates deserving of further study.

In the United States, colon cancer is the most prevalent malignancy and the leading cause of death among digestive system diseases.[1] To establish novel therapeutic and diagnostic strategies against this deadly disease, it is essential to understand its molecular pathology. One of the key molecular pathogenic elements underlying human cancer is inactivation of tumor suppressor genes. Inactivation of tumor suppressor genes is frequently due to promoter hypermethylation.[2] Therefore, cancer-specific promoter hypermethylation can itself serve as a valuable clue to uncover novel tumor suppressor genes.

In this context, we conducted a genome-wide search for novel targets of promoter hypermethylation in colon cancers by utilizing a 2-pronged microarray-based and *in silico* gene-filtering method. Herein, we report the results of this search and subsequent epigenetic validation. Seven novel genes were identified as targets of promoter hypermethylation-induced gene silencing in primary colon cancers. Most notably, 2 of these 7 novel genes were well-characterized gastrointestinal neuroendocrine and growth-regulatory peptide genes: *somatostatin* (*SST*) and the gene encoding precursor for neuroendocrine peptides such as substance P, *tachykinin-1* (*TAC1*).

Neuroendocrine peptides play essential roles in the regulation of gastrointestinal endocrine and exocrine secretion, motility, and mucosal immunity. Moreover, some neuroendocrine peptides, including SST, have been implicated in the modulation of human tumorigenesis by both direct and indirect means.[3,4] The current findings provide novel direct epigenetic evidence in human patients for the involvement of SST and TAC1 in the process of human colon tumor suppression.

Materials and Methods

Materials

Fifty-six primary human colon cancers, 22 noncancerous human colon mucosae, and 14 human colon cancer cell lines were included in this study. Colonic tissues were macroscopically dissected to enrich colonic mucosal layers or tumor cells. Genomic DNA and total RNA were extracted from snap-frozen tissues. Tumor microsatellite instability (MSI) status was determined based on 11 microsatellite markers as described

Abbreviations used in this paper: 5-aza-dC, 5-aza-2-deoxycitidine; ACTB, β-actin; CAV1, caveolin-1; AKAP12, A-kinase anchoring protei-12; ENG, endoglin; EI, expression index; MI, methylation index; MSI, microsatellite instability; MSI-H, microsatellite-unstable; MSI-L, low-level microsatellite instability; MSS, microsatellite-stable; MSP, methylation-specific PCR; SP, substance P; SST, somatostatin; TAC1, tachykinin-1; UTR, untranscribed region.

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Table 1. Colon Tissue Characteristics in Microarray and MSP Experiments

	Microarray		MSP	
	Primary cancers (n = 27)	Normal mucosae (n = 7)	Primary cancers (n = 34)	Normal mucosae (n = 17)
Age				
mean	66.9	63.8	67.1	65.1
maximum	91.0	70.0	82.0	82.0
minimum	36.0	50.0	44.0	46.0
S.D.	12.7	9.3	9.7	9.5
NA	0	3	0	5
P^a	.640		.532	
Sex				
F	20	0	8	6
M	21	4	26	9
NA	0	3	0	2
Location				
R	15		14	
L	12		19	
NA	0		1	
MSI status				
H	12		9	
L	0		7	
S	15		18	
Dukes stage				
A	3		4	
B	13		9	
C	9		10	
D	1		10	
NA	1		1	
Histologic differentiation				
WD	0		3	
MD	14		22	
MPD	6		4	
PD	6		4	
NA	1		0	

NOTE. MSI status: H, MSI-H; L, MSI-L; S, MSS.
Histologic differentiation: WD, well differentiated; MD, moderately differentiated; MPD, moderately to poorly differentiated; PD, poorly differentiated.
NA, not available.
[a]*P* Student *t* test *P* value for the comparison between primary cancers and normal mucosae.

previously.[5] Briefly, specimens with MSI at 30% or more of informative loci were labeled as microsatellite unstable (MSI-H), specimens showing MSI at less than 30% of informative loci were labeled as having low-level microsatellite instability (MSI-L), and specimens with no MSI were labeled as microsatellite stable (MSS). The clinicopathologic characteristics of the cases are summarized in Table 1. All colonic tissues were selected from our tissue repository based on the availability of a sufficient amount of high-quality nucleic acids (DNA and/or RNA), clinicopathologic data, and informative MSI data at more than 5 microsatellite loci including BAT25 and BAT26. This tissue repository contained surgically resected colonic tissues at the University of Maryland, Baltimore, and the Baltimore VA Hospital collected under institutional review board (IRB) approval and with informed consent. The current study was approved by the IRB at the University of Maryland at Baltimore.

5-Aza-2-deoxycitidine Treatment

Colon cancer cell lines HT29 and HCT116 were seeded at a density of 4×10^5 cells per 75-cm^2 culture flask and incubated for 24 hours in growth media. The cells were then treated with 5-aza-2-deoxycitidine (5-aza-dC) in either of the following conditions: (1) 10 μmol/L 5-aza-dC for 84 hours or (2) 1 μmol/L 5-aza-dC for 24 hours followed by 60 hours of 5-aza-dC-free growth. These 2 5-aza-dC treatment regimens were employed to obtain expression data under diverse cellular conditions: the former regimen focused on maximum DNA demethylation, and the latter regimen focused on moderate DNA demethylation to minimize damage to cell viability.

cDNA Microarray Analysis

Complementary DNA (cDNA) microarray analysis using an in-house 8064 human gene microarray was performed as described previously.[5] In brief, 30 μg of total RNA were amplified using a T7-based protocol, and 6 μg of the resulting amplified RNA (aRNA) were labeled with Cy5-dCTP. An aRNA pool of human cancer cell lines was used as the reference probe and labeled with Cy3-dCTP. The expression level of each gene was represented by the log-transformed Cy5-Cy3 intensity ratio, and global intensity-based normalization was performed.[6]