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Association of DNA Methylation and Epigenetic Inactivation of RASSF1A and Beta-Catenin with Metastasis in Small Bowel Carcinoid Tumors

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We analyzed promoter methylation of RASSF1A, CTNNB1, CDH1, LAMB3, LAMC2, RUNX3, NORE1A, and CAV1 using methylation-specific PCR in 33 cases of small bowel carcinoid with both matched primary and metastatic tumors. The methylation status of RASSF1A and CTNNB1 were also determined in six primary appendiceal carcinoid tumors. Two neuroendocrine cell lines, NCI-H727 and HTB-119, were analyzed for promoter methylation. Immunohistochemical analyses for RASSF1A and beta-catenin were performed in 28 matched primary and metastatic tumors. Western blot analysis for RASSF1A and beta-catenin was also performed. Normal enterochromaffin cells were unmethylated in all eight genes examined. RASSF1A and CTNNB1 were unmethylated in appendiceal carcinoids. Methylation of RASSF1A and CTNNB1 promoters was more frequent in metastatic compared to primary tumors ($p = 0.013$ and 0.004, respectively). The NCI-H727 and HTB-119 cells lines were methylated in the RASSF1A promoter region, and after treatment with 5-aza-2'-deoxycytidine (5-AZA), RASSF1A mRNA was expressed in both cell lines. Western blot results for RASSF1A and beta-catenin supported the methylation-specific PCR findings. The other six genes did not show significant differences. These results suggest that increased methylation of RASSF1A and CTNNB1 may play important roles in progression and metastasis of small bowel carcinoid tumors.

Key Words: Beta-catenin; carcinoid tumor; DNA methylation; epigenesis, genetic; gene expression; RASSF1A.

Introduction

Carcinoid tumors are rare slow-growing malignancies that can behave aggressively with development of metastatic disease leading to death. They occur most frequently in the gastrointestinal tract (67%), where they are most common in small bowel (25%), rectum (14%), and appendix (12%) *(1)*. Although rare, the incidence of gastrointestinal carcinoid tumors has been increasing in recent years, with 38.4 cases per one million persons in 1997. In comparison, the incidence was only 8.5 cases per one million persons in 1973 *(2)*. Small bowel carcinoids constitute 44.7% of carcinoids within the entire gastrointestinal tract *(2)*.

The mechanisms leading to carcinoid tumor development and progression are poorly understood. Wang et al. *(3)* found that allelic loss of chromosome 18q was present in 56–69% of ileal carcinoid tumors, and that ileal carcinoid tumors had distinctive clinicopathologic and genetic profiles. Recently, aberrant promoter methylation and epigenetic inactivation of tumor suppressor genes have been shown to be a common event in human cancers *(4,5)*. Promoter methylation is widely studied and may be a potential biomarker system for the diagnosis and detection of early cancers *(6)*. DNA methylation of tumor suppressor gene may also be related to tumor progression and metastasis. Recent studies have shown that epigenetic inactivation may be related to metastasis in some tumors *(7)*.

Promoter DNA methylation has been studied in lung carcinoids and other neuroendocrine tumors, including small-cell lung carcinoma and gastrointestinal carcinoids *(4,8)*. However, because of the small number of cases studied, the relationship between DNA promoter methylation and metastasis in small bowel carcinoids has not been well examined. We performed promoter methylation studies in a large series of small bowel carcinoids with both primary and metastatic tumors, analyzed the relationship between methylation and tumor development and progression. Many of the genes selected for study have been shown to be methylated in the promoter region in other tumors including gastrointestinal cancers, which are related to carcinoid tumors by location in the gastrointestinal tract. Our results suggest that RASSF1A

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and CTNNB1 methylation may have roles in tumor progression in midgut carcinoid tumors.

Results

CpG Island Methylation in Cell Lines and 5-AZA Treatment

RASSF1A was methylated in both NCI-H727 and HTB-119 cell lines (Fig. 1A); RASSF1A mRNA was undetectable in the two cell lines by RT-PCR. After 5-AZA treatment, RASSF1A mRNA was expressed (Fig. 1B). RUNX3 was methylated in NCI-H727 and LAMB3 was methylated in HTB-119, but none of the other genes showed methylation in these two cell lines. Western blot analysis showed loss of RASSF1A expression in the two cell lines, while beta-catenin was highly expressed (Fig. 1C).

CpG Island Methylation in Normal EC Cells and Carcinoid Tumors

MS-PCR performed for the eight genes from LCM-collected normal EC cells showed that all genes were unmethylated (Fig. 2). All tumors studied were methylated in at least one gene. The highest methylation frequency was in LAMB3 and the lowest in CAV1. Promoter methylation of RASSF1A was more frequent in the metastatic (84.8%) compared with the primary tumors (60.6%) ($p = 0.013$) (Figs. 2A,C). CTNNB1 was also more frequently methylated in the metastatic (57.6%) than in primary tumors (27.3%) ($p = 0.004$) (Figs. 2B,C). The other six genes did not show significant differences in methylation between the primary and metastatic tumors. There was no methylation of RASSF1A and CTNNB1 in the six appendiceal carcinoid tumors (data not shown). After LCM and MS-PCR analysis of two tumors, only the tumor cells were methylated in the RASSF1A and CTNNB1 promoter region, while the stromal cells were unmethylated (data not shown).

Immunohistochemical Analysis of Proteins

The results of immunohistochemical analysis for RASSF1A, beta-catenin, and E-cadherin proteins are shown in Table 1 and Fig. 3. There was decreased expression of RASSF1A in metastatic compared to primary tumors, but the differences were not significant ($p = 0.444$) (Figs. 3A,B, Table 1). Beta-catenin showed predominantly membrane staining and the staining was significantly lower in metastatic than in primary tumors ($p = 0.004$) (Figs. 3C,D, Table 1). There was focal weak staining for nuclear beta-catenin, but the differences between the primary and metastatic tumors were not significant.

Western Blot Analysis

Western blot analysis showed that there was significantly lower expression of RASSF1A in matched metastatic tumors (12.5%) than in primary tumors (56.2 %) (Fig. 4A, $p = 0.023$). Beta-catenin protein levels were also decreased in the metastatic (13.3%) compared to the primary tumors (60%) ($p = 0.023$) (Fig. 4B).

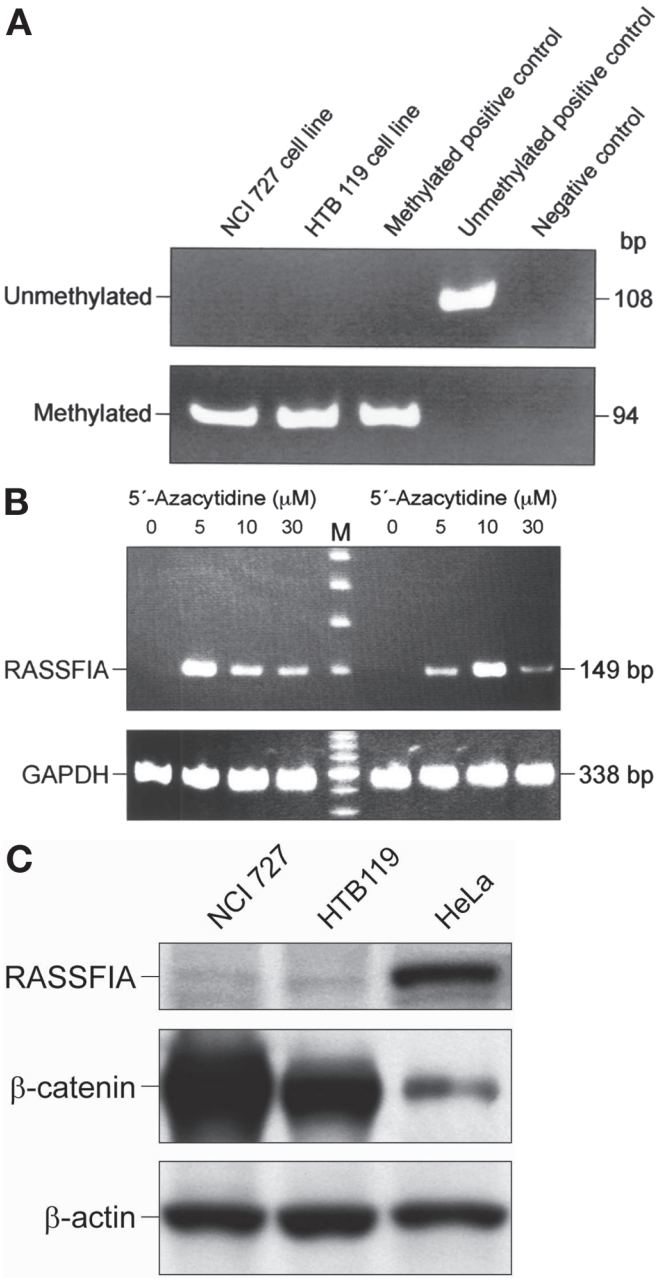

Fig. 1. MS-PCR analysis and expression of RASSF1A and beta-catenin in neuroendocrine cell lines and 5-AZA treatment. (**A**) MS-PCR analysis of NCI-H727 and HTB-119 cell lines with unmethylated (top) and methylated (bottom)-specific primers were shown. (**B**) RT-PCR showing RASSF1A mRNA expression in the NCI-H727 (left) and HTB-119 (right) cell lines before and after 5-AZA treatments. (**C**) Western blot showed RASSF1A and beta-catenin protein expression in the two cell lines. HeLa cells were used as control.

DNA Sequencing

No mutations were identified in beta-catenin exon 3 and K-RAS exons 12 and 13 (data not shown).

Discussion

Analysis of promoter methylation and gene expression in primary and metastatic carcinoid tumors showed signifi-

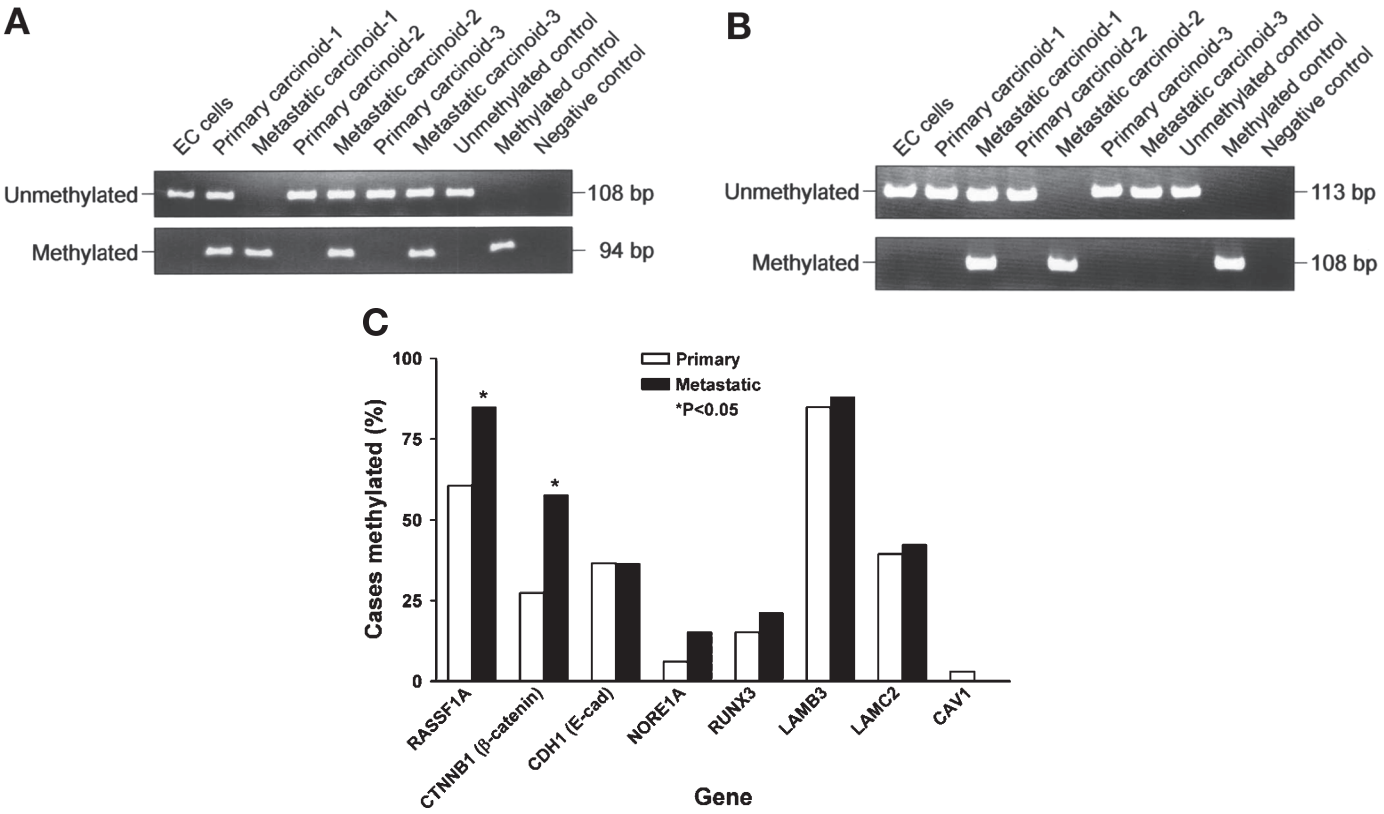

Fig. 2. MS-PCR in normal EC cells, primary and metastatic carcinoid tumors (33 cases). Normal EC cells were unmethylated in all genes. (**A**) RASSF1A methylation was detected in the metastatic tumors in three cases while two of the primary tumors were unmethylated. (**B**) Beta-catenin methylation was present in two metastatic tumors while all three primary tumors were unmethylated. (**C**) Promoter methylation frequency in small bowel carcinoid tumors showed significant differences between primary and metastatic tumors for RASSF1A and CTNNB1.

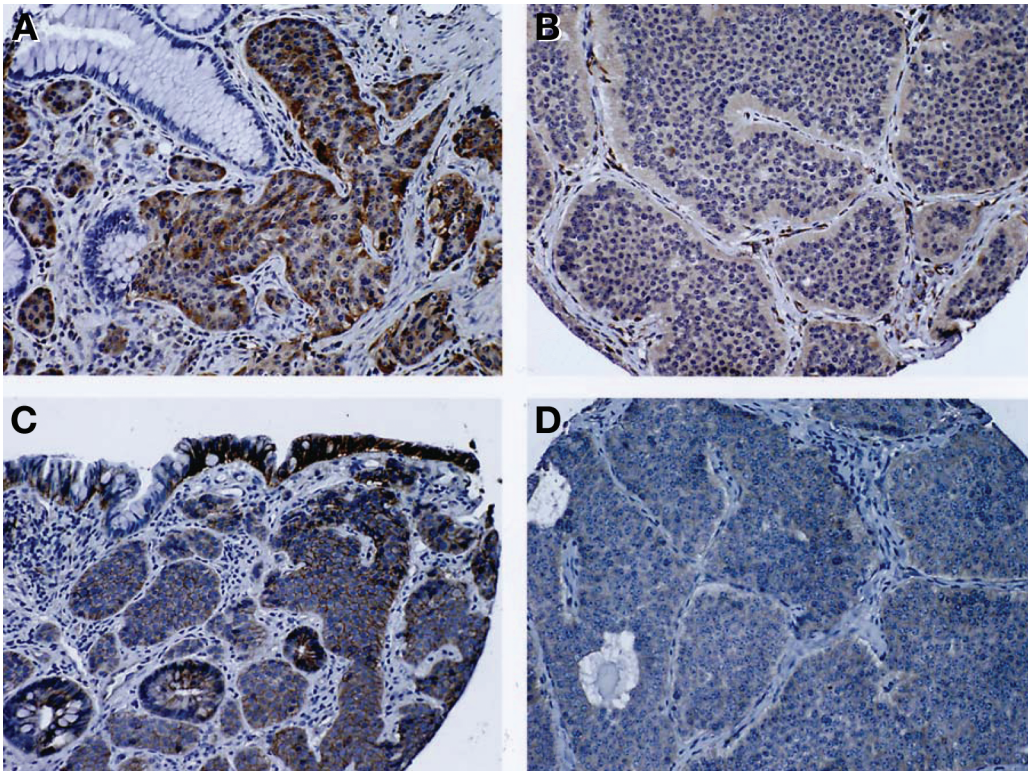

Fig. 3. Immunohistochemical analysis of RASSF1A and beta-catenin in carcinoid tumors. RASSF1A was expressed more strongly in the primary tumors (**A**) compared to the liver metastasis (**B**). Beta-catenin showed stronger membrane and cytoplasm staining in the primary (**C**) compared to the metastatic tumors (**D**) (200×).

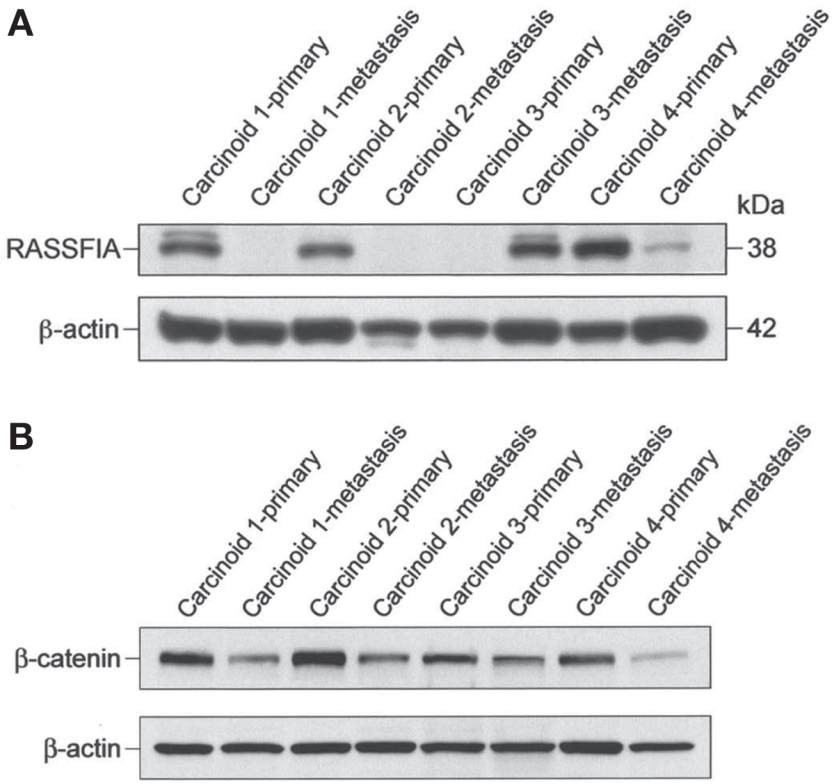

Fig. 4. Western blot of RASSF1A and beta-catenin in primary and metastatic carcinoid tumors. (**A**) RASSF1A protein expression was significantly higher in primary compared to metastatic tumors. (**B**) Beta-catenin protein expression was generally higher in primary compared to metastatic tumors.

Table 1
Immumohistochemical Staining for RASSF1A and Beta-Catenin in Primary and Metastatic Carcinoid Tumors

	Primary	Metastatic	
	Score		
Antibody	Mean ± SEM[b]	Mean ± SEM	*p* value[a]
RASSF1A	2.8 ± 0.11	2.5 ± 0.89	0.444
Beta-catenin (membrane)	3.1 ± 0.14	2.5 ± 0.19	0.004

[a]Analyzed by *t*-test.
[b]Standard error of the mean.
The mean ± SEM represents the immunohistochemical grading intensity which ranged from 0 (negative) to 4+ (very strong staining).

cant differences between the primary and metastatic tumors for two of the eight genes studied, including RASSF1A and CTNNB1, suggesting that epigenetic changes in these genes may have a role in tumor progression supported by higher methylation of RASSF1A and CTNNB1 in metastatic compared to matching primary carcinoid tumors. Analysis of six appendiceal carcinoids that did not have metastatic disease showed that all cases were unmethylated for RASSF1A and CTNNB1. Appendiceal carcinoids rarely metastasize, so the presence of the unmethylated RASSF1A and CTNNB1 genes would support the role of methylation of these genes in carcinoid tumor progression. Treatment of two neuroendocrine cell lines which were methylated for RASSF1A with 5-AZA led to expression of RASSF1A mRNA, indicating that methylation inhibited gene expression.

RASSF1A tumor suppressor gene located on chromosome 3p21.3 has an important role in RAS signaling by binding to RAS, transmitting signals from receptor tyrosine kinases to the nucleus, regulating cell growth, survival, and differentiation. Activated RASSF1A inhibits cyclin D1 accumulation, blocks cell cycle progression, and suppresses cell proliferation. RASSF1A also activates MST1, an apoptotic protein kinase, leading to cell apoptosis *(9,10)*. Ectopic expression of RASSF1A in cell lines caused suppression of the malignant phenotype both in vivo and in vitro. RASSF1A was frequently inactivated in many types of human cancers by aberrant promoter methylation, including small-cell lung, non-small-cell lung, breast, ovarian, gastric, prostate, colon, and thyroid carcinomas *(11–13)*. Inactivation of RASSF1A has also been associated with advanced tumor stage and poor prognosis *(14)*. However, some studies have not found a correlation between RASSF1A methylation and survival *(15)*. Wild-type RASSF1A was found to be associated with microtubules by immunofluorescence, and the microtubule stability induced by RASSF1A probably has a role in cell adhesion and motility *(16)*. RASSF1A methylation results in decreased expression of RASSF1A

protein, which leads to release of inhibition of cyclin D1, and accumulated cyclin D1 can then promote cell proliferation. We detected a significant decrease in RASSF1A protein expression in metastatic carcinoids by Western blotting, but not by TMA analysis, suggesting that the Western blot was more sensitive and correlated with the increase methylation of the RASSF1A gene. Our laboratory reported RASSF1A methylation in small bowel carcinoid tumors, and observed a higher degree of concordance between loss of RASSF1A expression and methylation in preliminary studies *(17,18)*. Liu et al. subsequently reported a correlation of RASSF1A methylation and metastasis of carcinoid. This observation is consistent with our findings *(8)*. Normal EC cells were unmethylated, while primary carcinoids showed a high frequency of methylation, suggesting that RASSF1A is associated with tumorigenesis. In separately collected tumor cells and adjacent stromal cells using laser capture microdissection (LCM), only the tumor cells showed methylation, indicating that the methylation we observed was from tumor cells and not from the stromal cells. Toyooka et al. *(19)* found a high frequency of RASSF1A methylation in pulmonary neuroendocrine tumors. Recently, Pizzi et al. *(20)* evaluated 62 endocrine tumors from different locations of the gastrointestinal tract, and found that RASSF1A methylation and 3p21.3 LOH occurred only in foregut, but not in midgut or hindgut carcinoid tumors, which is different from our observations. Their study only examined a small number of primary ileal tumors and no metastatic tumors, which may explain the differences between their findings and ours.

Mutation analysis of K-RAS in exon 2 showed that all tumors studied were wild-type, suggesting that epigenetic alteration in RASSF1A may be more common in the pathogenesis of small bowel carcinoid than mutations of K-RAS.

The human beta-catenin gene (CTNNB1), located on chromosomal region 3p22-p21.3, is in the same chromosomal region as RASSF1A. Beta-catenin links E-cadherin and actin cytoskeleton through interaction with alpha-catenin in adherent junctions. Abnormalities of cell adhesion molecules are known to play an important role in invasion and metastasis of cancer cells through loss of cell-to-cell adhesion. LOH and deletion of the short arm of chromosome 3 have been observed at high frequency in a variety of human tumors *(21)*. In addition to its critical role in cellular adhesion, beta-catenin also functions in the Wnt signaling pathway *(22)*. There are few studies on the methylation status of beta-catenin *(23,24)*, so the role of beta-catenin methylation in the pathogenesis and metastasis of carcinoids is unclear. Our results showed a high frequency of beta-catenin methylation both in primary and metastatic carcinoids, and methylation was more common in metastatic tumors. These findings correlated with the immunohistochemical findings of protein expression. Although nuclear beta-catenin accumulation is usually due to mutation of degradation sensitive sites which activates wnt signaling allowing beta-catenin to act as an oncogene, our finding with increased methylation of the beta-catenin promoter region suggests that other mechanisms may be operating when beta-catenin expression decreases. Our study is in agreement with Ebert et al. *(23)*, who found loss of beta-catenin in primary gastric cancers. It is possible that decreased expression of beta-catenin by methylation impairs cell-to-cell adhesion with tumor cells detaching more easily and transported to a distant site. Overexpression of beta-catenin in some tumors results from gain-of-function mutations in exon 3. In our study, there were no mutations in exon 3 of beta-catenin, suggesting that mutation of exon 3 of beta-catenin is uncommon in carcinoid tumors *(25,26)*.

Other genes analyzed for methylation in small bowel carcinoid tumors showed no significant differences between the primary and metastatic tumors, including NORE1A, CDH1, LAMB3, LAMC2, RUNX3, and CAV1.

In summary, this study suggests that RASSF1A and CTNNB1 may have roles in tumor progression and metastasis of small bowel carcinoid tumors. Promoter methylation of RASSF1A and CTNNB1 genes was more frequent in metastatic than in primary tumors using matching primary and metastatic cases. There was generally good agreement between loss of protein expression and methylation for RASSF1A and CTNNB1. Both RASSF1A and CTNNB1 are located in the same region of chromosome 3p22-21.3. This region often has deletions and LOH, and may be important for the development of small bowel carcinoid tumors.

Materials and Methods

Clinical Samples

Genomic DNA was extracted from both primary ileal carcinoid ($n = 33$) and the matched metastatic tumors to the liver ($n = 25$) or lymph nodes ($n = 8$). Primary appendiceal carcinoid tumors from six patients without metastases were also studied. Small bowel carcinoids were also used to construct tissue microarray (TMA) ($n = 28$) and for protein extraction ($n = 16$). Tumor tissues were from patients who had surgery between 1990 and 2006 at Mayo Clinic, Rochester, MN. Institutional review board permission was obtained for the study. Normal enterochromaffin cells were collected from non-neoplastic ileal tissues of six pooled cases by immunostaining for chromogranin A, followed by LCM. Before DNA extraction and methylation study, hematoxylin and eosin staining was done in tumors; all samples had 70% or more tumor cells in the sections.

Cell Lines

Two neuroendocrine cell lines, NCI-H727 and HTB-119, were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 with 10% FBS in 5% CO_2 at 37°C.

DNA Extraction

Genomic DNA was extracted as described previously from tumor tissues *(17,27)*, two cell lines, and LCM-collected

Table 2
Primers for MS-PCR Analysis

Name	Genbank Acc.		Primers	Length (bp)	Source
		U-PCR:	TTTGGTTGGAGTGTGTTAATGTG		
			CAAACCCCACAAACTAAAAACAA	108	Lo *(11)*
		M-PCR:	GTGTTAACGCGTTGCGTATC		
			AACCCCGCGAACTAAAAACGA	94	
NORE1A	NM_031437	U-PCR:	ATTTATATTTGTGTAGATGTTGTTTGGTAT		
			ACTTTAACAACAACAACTTTAACAACTACA	215	Hesson *(12)*
		M-PCR:	CGTCGTTTGGTACGGATTTTATTTTTTTCGGTTC		
			GACAACTTTAACAACGACGACTTTAACGACTACG	202	
CTNNB1	AY463360	U-PCR:	TTGGGAATTTGTAGATTAGTGATGG		
			AAAAAACAAAAATTCAAAACCAAAACAACA	113	This study
		M-PCR:	GGTCGGGAATTCGTAGATTAGCGAC		
			GAAAATTCGAAACCAAAACGACGAC	108	
CDH1	L34545	U-PCR:	TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT		
			ACACCAAATACAATCAAATCAAACCAAA	120	House *(13)*
		M-PCR:	TGTAGTTACGTATTTATTTTTAGTGGCGTC		
			CGAATACGATCGAATCGAACCG	112	
RUNX3	AL023096	U-PCR:	AAGTGGGAAAGTAGAAGTGGTG		
			CCAAACAAACTACAAACAACCA	126	Kim,TY *(14)*
		M-PCR:	TATTCGTTAGGGTTCGTTCGT		
			AAACAACCACGAAAAACGAC	120	
LAMB3	AL031316	U-PCR:	GATTGATTAATTTATTTGTTTAGTTTT		
			CAAATCTCAAAAATCTAACAACCA	92	Sathyanarayana *(15)*
		M- PCR:	ATCGATTAATTTATTTGTTTAGTTTC		
			GAATCTCAAAAATCTAACAACCG	90	
LAMC2	AL354953	U-PCR:	TTATTAATTGAGGTGTTGGGTAGTGA		
			CCAACCAAAACACAAACATAACAAAACCAA	86	This study
		M-PCR:	TTATTAATCGAGGCGTCGGGTAGCG		
			CCAAAACGCAAACATAACGAAACCG	82	
CAV-1	AJ133269	U-PCR:	TAGTGTGGGAGAAATGTTTTTATTTGT		
			TTAACCCAAACATATACTACACCAAA	114	This study
		M-PCR:	TAGCGCGGGAGAAACGTTTTTATTC		
			TAACCCGAACGTATACTACGCCGAA	113	

normal EC cells. Briefly, paraffin-embedded specimens were cut at 10 μm × 5 pieces, deparaffined with xylene, and dehydrated with ethanol. The tissue specimens were then incubated in 2 mL solution (pH 7.4) containing 10 mmol Tris-HCl, 25 mmol EDTA, 10 mmol NaCl at 37°C for 30 min (TEN). Proteinase K (Roche Diagnostics, Alameda, CA) was added to the solution for a final concentration of 0.2 mg/mL, and incubated overnight at 55°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (Invitrogen, Carlsbad, CA), ethanol-precipitated, air-dried, and resuspended in sterile water. For fresh tissue, samples were directly minced, digested with TEN solution and Proteinase K, and used for DNA extraction. For cell lines, cultured cells were harvested and used for DNA extraction. The DNA concentration was determined by optical densitometry with a spectrophotometer.

Bisulfite Modification

Bisulfite modification was done on genomic DNA samples as previously reported *(27)*. Briefly, 2 μg of genomic DNA from each sample was bisulfite-modified using the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. One microgram of DNA from human placenta (Sigma, St. Louis, MO) or/and HeLa cells (ATCC) was used as the unmethylated control and 1 μg of CpGenome Universally Methylated DNA (Chemicon International, Temecula, CA) was used as methylated control and were treated concurrently with the tumor samples. The bisulfite-modified DNA was eluted in 10 μL of elution buffer from the kit and stored at –20°C. One microliter of the bisulfite-modified DNA was used for each PCR reaction.

Methylation-Specific PCR (MS-PCR)

PCR primers were used from previously published studies *(28–32)* or designed by using the Oligo-6.61 Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, CO). The primers used in this study included RASSF1A, CTNNB1, CDH1, LAMB3, LAMC2, RUNX 3, NORE1A, and CAV 1 (Table 2).

Both unmethylated (U)-specific and methylated (M)-specific set of primers (forward and reverse) were designed based on the positive strand of the bisulfite-converted DNA and spanned the CpG island within the promoter region.

One microliter of bisulfite-modified DNA from each sample was amplified independently using the U- and M-specific primers in a 25 μL total volume reaction. Each PCR reaction contained a final concentration of 0.2 μ*M* of each primer, 0.2 m*M* dNTPs (Roche), 1X Easy-A reaction buffer, 1.25 units of Easy-A high-fidelity PCR cloning enzyme (Stratagene, La Jolla, CA). All PCR reactions were done using a Gene Amp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA). Ten microliters of each PCR product was run on a 10% Tris-borate EDTA ready gel (Bio-Rad, Hercules, CA) in 1X Tris-borate EDTA. The gel was briefly stained with 0.1 mg/mL ethidium bromide and visualized under UV light.

Beta-Catenin and K-RAS Mutation Analysis

Exon 3 mutation status of beta-catenin was analyzed in 22 tumors (13 primary and 9 metastatic) to determine the relationship between gene mutations and promoter methylation. The primers used were: sense, 5'-GCTGATTTGAT GGAGTTGGA and anti-sense, 5'-GCTACTTGTTCTTG AGTGAA *(33)*. To evaluate the relationship between RAS mutations and RASSF1A promoter methylation, 32 tumors (20 primary and 12 metastatic) were used for K-RAS mutation analysis in codons 12 and 13. The primers used were: sense, 5'-AGGCCTGCTGAAAATGACTGAATA and antisense, 5'-CTGTATCAAAG AATGGTCCTGCAC *(34)*.

Two separate PCR reactions were amplified for each case and used for cloning. Both 25 μL PCR reactions were pooled and concentrated using a Microcon YM-100 centrifugal filter device (Millipore, Bedford, MA). The concentrated product was inserted into the pCR 4-TOPO vector and transformed into TOPO-10 chemically competent cells using a TOPO-TA cloning kit for sequencing (Invitrogen) following the manufacturer's instructions. Transformed cells were plated on LB agar containing 50 μg/μL ampicillin (Invitrogen) and incubated overnight at 37°C. Three individual colonies were selected and each inoculated into 5 mL LB broth containing 50 μg/mL ampicillin (Invitrogen) and grown overnight at 37°C. The insert-containing plasmid DNA was then extracted from the cells using the Qua-Prep spin mini-prep kit (Qiagen, Valencia, CA) and resuspended in 30 μL of EB buffer provided by the kit. Four microliters of the purified plasmid was combined with 1.6 p*M* M-13 reverse primer (Invitrogen) and automatically sequenced using the 3730 XL DNA analyzer (Applied Biosystems). A minimum of three clones were analyzed for each experiment.

Demethylation Studies

NCI-H727 and HTB-119 cells were plated in six well plates at 200,000 cells/well. On the second day, cells were treated with 5-aza-2'-deoxycytidine (5-AZA, Sigma) at concentration 0, 5 μ*M*, 10 μ*M*, 15 μ*M*, and 30 μ*M* for 72 h (NCI-H727) or 48 h (HTB-119). Cells were then collected by trypsinization. Total RNA was extracted using TRIzol (Life Technologies) as previously described *(27)*. The RASSF1A mRNA expression was detected by RT-PCR as previously described. The primers used were: sense, 5'-GCTCGTCTGC CTGGACTGTT and antisense, 5'-GGGCATTGTACTCCT TGATCTT *(35)*. GAPDH was used as internal control. Primers used were: sense, 5'-TCACCATCTTCCAGGAGCGA GA and antisense, 5'-CTTCTGGGTGG CAGTGATG *(36)*.

TMA and Immunohistochemistry

Tissue microarrays were constructed as previously described *(37)*. Briefly, 0.6 mm cores from selected blocks of formalin-fixed, paraffin-embedded tissue were taken in triplicate and placed in the TMA block and liver tissues were also placed in the arrays for orientation. TMA sections cut at 4 μm were used for immunohistochemical staining. Antigen retrieval was performed by microwaving in 0.1 m*M* citrate buffer at pH 6.0 for 5 min. Antibodies used for TMA slides included RASSF1A (1:250) from eBiosciences (Minneapolis, MN) and beta-catenin (1:500). Immunostaining was performed with avidin–biotin–peroxidase complex method (Vector Corp., Burlingame, CA) and diaminobenzidene was used as chromogen. Hematoxylin was used for counterstaining RASSF1A, but not for beta-catenin.

Evaluation of immunohistochemical results was performed by grading the intensity of staining on a scale of 0–4 with 0 for negative staining, 1+ for weak staining, 2+ for moderate staining, 3+ for strong staining, 4+ for very strong staining. The TMA triplicates were then averaged. Separate nuclear and membrane staining was analyzed for beta-catenin. Normal EC cells were used as positive control, and substitution of the primary antibody with normal serum was used as the negative control for immunohistochemical staining.

Laser Capture Microdissection

LCM was performed following immunostaining for chromogranin A (Neomarkers) from non-tumor areas of small bowels resected during tumor operations in six patients as previously reported *(38)*. Briefly, paraffin-embedded blocks were cut at 10 μm and attached to uncharged glass slides. After immunostaining, the slides were dehydrated with 95% and 100% ethanol, and then immersed in xylene for 10 min, and air-dried. LCM was done with a PixCell II Laser Capture Microdissection System (Arcturus Engineering, Inc., Mountain View, CA). The chromogranin A immunopositive normal EC cells were identified easily under microscope and captured onto a thermoplastic polymer-film-coated cap by a one-step transfer. An infrared laser with 60 mW power and 7.5 μm diameter was used to capture cells of interest. The laser melted the ethylene vinyl acetate from a plastic film directly onto the target cell, and embedded the captured cells. A total of approx 10,000 cells were captured and the

cells were stored in −70°C freezer until DNA extraction. To determine that the methylation was from tumor cells but not from adjacent stromal cells or normal cells within the samples, we performed LCM on two tumors that were known methylated in RASSF1A and CTNNB1, collected the tumor cells, and stromal cells separately, extracted DNA and checked the methylation status.

Western Blot

Proteins from 32 frozen tissues (16 primary and 16 metastatic tumors from the same patients) and the 2 cell lines were extracted in the presence of protease inhibitors. Twenty-five micrograms of protein was used for one-dimensional sodium dodecyl sulfate polycrylamide gel electrophoresis in a 12% gel. The electrophoresed protein was transferred to a polyvinylidene difluoride membrane (BioRad, Richmond, CA) and subjected to immunoblot analysis with an antibody to RASSF1A (1:500, $n = 16$ cases) and beta-catenin (1:1000, $n = 15$ cases). Proteins on membranes were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Beta-actin was used as an internal control *(11)*.

Data Analysis

Frequencies of methylation of different gene groups in primary and metastatic tumors were compared using McNemar's paired Chi square test. Student's *t*-test was used to compare immunohistochemical staining scores between primary and metastatic tumors. Western blot data were also analyzed by McNemar's paired Chi square test. All statistical tests were two sided, probability values of $p < 0.05$ were regarded as significant.

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