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IGFBP3 Promoter Methylation in Colorectal Cancer: Relationship with Microsatellite Instability, CpG Island Methylator Phenotype, and p53[1]

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Abstract

Insulin-like growth factor binding protein 3 (IGFBP3), which is induced by wild-type p53, regulates IGF and interacts with the TGF-β pathway. *IGFBP3* promoter methylation may occur in colorectal cancer with or without the CpG island methylator phenotype (CIMP), which is associated with microsatellite instability (MSI) and *TGFBR2* mutation. We examined the relationship between *IGFBP3* methylation, p53 expression, CIMP and MSI in 902 population-based colorectal cancers. Utilizing real-time PCR (MethyLight), we quantified promoter methylation in *IGFBP3* and eight other CIMP-high–specific promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*). *IGFBP3* methylation was far more frequent in non–MSI-high CIMP-high tumors (85% = 35/41) than in MSI-high CIMP-high (49% = 44/90, *P* < .0001), MSI-high non–CIMP-high (17% = 6/36, *P* < .0001), and non–MSI-high non–CIMP-high tumors (22% = 152/680, *P* < .0001). Among CIMP-high tumors, the inverse relationship between MSI and *IGFBP3* methylation persisted in p53-negative tumors (*P* < .0001), but not in p53-positive tumors. *IGFBP3* methylation was associated inversely with *TGFBR2* mutation in MSI-high non–CIMP-high tumors (*P* = .02). In conclusion, *IGFBP3* methylation is inversely associated with MSI in CIMP-high colorectal cancers, and this relationship is limited to p53-negative tumors. Our data suggest complex relationship between global genomic/epigenomic phenomena (such as MSI/CIMP), single molecular events (e.g., *IGFBP3* methylation, *TP53* mutation, and *TGFBR2* mutation), and the related pathways.

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Introduction

Insulin-like growth factor binding protein 3 (IGFBP3) is the main carrier of insulin-like growth factors (IGFs) in the circulation, where this complex regulates biologic function of IGFs [1]. IGFBP3 has been shown to regulate both cell growth and death, independently of its interaction with IGFs [2,3]. *IGFBP3* promoter methylation and gene silencing are observed in human cancers including colorectal cancer [4,5], and have been associated with poor clinical outcome in lung and ovarian cancers [6,7]. IGFBP3 is induced by wild-type p53 [8], and promoter methylation at the p53 regulatory element causes gene silencing resistant to p53 [9]. IGFBP3 enhances the p53-dependent apoptotic response of colorectal adenoma cells to DNA damage [10]. IGFBP3 is known to interact with the transforming growth factor–beta (TGF-β) pathway [11–13].

Transcriptional inactivation by cytosine methylation at promoter CpG islands of tumor suppressor genes is an important mechanism in human carcinogenesis [14]. A number of tumor suppressor genes can be silenced by promoter methylation in colorectal cancers [14]. A subset of colorectal cancers exhibit widespread promoter CpG island methylation, which is referred to as the CpG island methylator phenotype (CIMP) [15]. CIMP-high colorectal tumors have a distinct clinical, pathologic, and molecular profile, such as associations with proximal tumor location, female, poor differentiation, *BRAF* mutation,

Abbreviations: *BAX*, BCL2-associated X protein; *CACNA1G*, calcium channel, voltage-dependent, T type alpha-1G subunit; *CDKN1A*, cyclin-dependent kinase inhibitor 1A (p21, CIP1); *CDKN2A*, cyclin-dependent kinase inhibitor 2A (p16, INK4A); CIMP, CpG island methylator phenotype; *CRABP1*, cellular retinoic acid binding protein 1; *CTNNB1*, β-catenin; *IGF2*, insulin-like growth factor 2; *IGFBP3*, insulin-like growth factor binding protein 3; MSI, microsatellite instability; MSI-H, microsatellite instability–high; MSI-L, microsatellite instability–low; MSS, microsatellite stable; *NEUROG1*, neurogenin 1; PCR, polymerase chain reaction; PMR, percentage of methylated reference (degree of methylation); *RUNX3*, runt-related transcription factor 3; *SOCS1*, suppressor of cytokine signaling 1; TGF, transforming growth factor; *TGFBR2*, transforming growth factor β receptor type 2; TMA, tissue microarray; *TP53*, tumor protein p53

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wild-type tumor protein p53 (*TP53*), and inactive WNT/*CTNNB1* (β-catenin) [16–24], independent of microsatellite instability (MSI) status [19–23]. In addition, CIMP-high in microsatellite instability–high (MSI-H) colorectal cancer is correlated with the transforming growth factor β receptor type 2 gene (*TGFBR2*) mononucleotide mutation [25]. Molecular classification of colorectal cancer based on CIMP and MSI is increasingly important [26,27] because MSI and CIMP represent global genomic and epigenomic phenomena, respectively, in tumor cells, and largely determine pathologic and molecular features of colorectal cancer [27].

In this study, using quantitative DNA methylation analysis (MethyLight) and a large number of population-based colorectal cancers, we have examined the relationship between *IGFBP3* promoter methylation and various molecular features in colorectal cancer, including MSI, CIMP, p53, and mutations in *TGFBR2* and BCL2-associated X protein (*BAX*). Discovering molecular correlates is important in cancer research, because it may: 1) provide clues to pathogenesis; 2) propose or support the existence of a new molecular subtype; 3) alert investigators to be aware of potential confounding in association studies; and 4) suggest surrogate markers in clinical or research settings [27].

Materials and Methods

Study Group

We used the databases of two large prospective cohort studies: the Nurses' Health Study (N = 121,700 women followed since 1976) [28] and the Health Professionals Follow-Up Study (N = 51,500 men followed since 1986) [29]. Informed consent was obtained from all participants before inclusion in the cohorts. A subset of the cohort participants developed colorectal cancers during prospective follow-up. Thus, these colorectal cancers represented population-based, relatively unbiased samples (compared to retrospective or single-hospital–based samples). Previous studies on the cohorts have described baseline characteristics of cohort participants and incident colorectal cancer cases, and confirmed that our colorectal cancer cases were well-represented as a population-based sample [28,29]. We collected paraffin-embedded tissue blocks from hospitals where cohort participants with colorectal cancers had undergone resections of primary tumors. We excluded cases if adequate paraffin-embedded tumor tissue was not available at the time of the study. As a result, a total of 902 colorectal cancer cases (405 from men's cohort and 497 from women's cohort) were included. Among our cohort studies, there was no significant difference in demographic features between cases with tissue available and those without available tissue [30]. Many of the cases have been previously characterized for status of CIMP, MSI, *KRAS*, and *BRAF* [23]. However, no tumor has been examined for *IGFBP3* methylation in our previous studies. Tissue collection and analyses were approved by the Dana-Farber/Harvard Cancer Center and Brigham and Women's Hospital Institutional Review Boards.

Histopathologic Evaluations

Hematoxylin and eosin (H&E)–stained tissue sections were examined under a light microscope by one of the investigators (S.O.) blinded from clinical and other laboratory data as previously described [22]. The following eight features were evaluated: 1) the presence and extent of extracellular mucin were categorized as negative (no mucin), < 50%, or ≥50% of the tumor volume; 2) the presence and extent of signet ring cells were categorized as negative (no signet ring cells), < 50%, or ≥ 50% of the tumor volume; 3) degree of tumor differentiation was categorized as well/moderate (≥ 50% gland formation) *versus* poor (< 50% gland formation); 4) to 6) the degree of Crohn's-like lymphoid reaction (defined as transmural lymphoid aggregates), the degree of a peritumoral lymphocytic reaction (defined as a discrete lymphoid infiltrate surrounding tumor cell nests), and the degree of tumor-infiltrating lymphocytes were graded as absent/mild *versus* moderate/severe; 7) the extent of extraglandular tumor necrosis was graded as < 20% *vs* ≥ 20%; and 8) the type of tumor border was categorized as circumscribed *versus* infiltrative.

Genomic DNA Extraction and Whole Genome Amplification

Genomic DNA was extracted from dissected tumor tissue sections using QIAmp DNA Mini Kit (Qiagen, Valencia, CA) as previously described [31]. Normal DNA was obtained from colonic tissue at resection margins. Whole genome amplification of genomic DNA was performed by polymerase chain reaction (PCR) using random 15-mer primers for subsequent MSI and loss of heterozygosity (LOH) analyses and *KRAS* and *BRAF* sequencing [31]. Previous studies by us and others showed that whole genome amplification did not significantly affect subsequent genetic analysis [31,32].

Analyses for MSI and 18q LOH

Methods to analyze for MSI and *TGFBR2* mutation have been previously described [25,33]. In addition to the recommended MSI panel consisting of D2S123, D5S346, D17S250, BAT25, and BAT26 [34], we also used BAT40, D18S55, D18S56, D18S67, and D18S487 (i.e., 10-marker panel) [33]. A high degree of MSI (MSI-H) was defined as the presence of instability in ≥ 30% of the markers. A low degree of MSI (MSI-L) was defined as the presence of instability in < 30% of the markers, and microsatellite stable (MSS) tumors were defined as tumors without an unstable marker. PCR primers for *BAX* mononucleotide repeat were: BAX-F, FAM–5′–atc cag gat cga gca ggg cg–3′; BAX-R, 5′–act cgc tca gct tct tgg tg–3′. PCR cycles consisted of initial denaturing at 94°C for 2 minutes, followed by 45 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 2 minutes. PCR products were analyzed by ABI 3730 (Applied Biosystems, Foster City, CA). The presence of a peak at an altered size in tumor DNA compared to normal DNA was interpreted as positivity for *BAX* mutation.

For 18q LOH analysis using microsatellite markers D18S55, D18S56, D18S67, and D18S487, we duplicated PCR reaction and electrophoresis in each sample to exclude allele dropouts

of one of two alleles [33]. LOH at each locus was defined as 40% or greater reduction of one of two allele peaks in tumor DNA relative to normal DNA. Overall 18q LOH positivity was defined as the presence of one or more markers with LOH, and overall 18q LOH negativity as the presence of two or more informative markers and the absence of LOH in all informative markers.

Sequencing of KRAS and BRAF

Methods of PCR and sequencing targeted for *KRAS* codons 12 and 13, and *BRAF* codon 600 have been previously described [31,35]. Pyrosequecing was performed using the PSQ96 HS System (Biotage AB and Biosystems, Uppsala, Sweden) according to the manufacturer's instructions.

Real-Time PCR (MethyLight) for Quantitative DNA Methylation Analysis

Sodium bisulfite treatment on genomic DNA was performed as previously described [36]. Real-time PCR to measure DNA methylation (MethyLight) was performed as previously described [37]. Using ABI 7300 (Applied Biosystems), we examined *IGFBP3* promoter and eight other CIMP-specific promoters [calcium channel, voltage-dependent, T type alpha-1G subunit (*CACNA1G*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) (*p16*), cellular retinoic acid binding protein 1 (*CRABP1*), insulin-like growth factor 2 (*IGF2*), *MLH1*, neurogenin 1 (*NEUROG1*), runt-related transcription factor 3 (*RUNX3*), and suppressor of cytokine signaling 1 (*SOCS1*)] [20,24]. We have shown that these eight markers are sensitive and specific markers for CIMP-high [23]. The collagen 2A1 gene (*COL2A1*) was used to normalize for the amount of input bisulfite-converted DNA [36]. The primers and probe for *IGFBP3* were (bisulfite-converted nucleotides are in italics): IGFBP3-F, 5′–G***T***T ***T***CG GGC GTG AG***T*** ACG A–3′ (GenBank No. M35878, nucleotides 1692–1710); IGFBP3-R, 5′–G***AA*** TCG ***A***CG CA***A*** ***A***CA CG***A*** CT***A*** C–3′ (GenBank No. M35878, nucleotides 1789–1810); and IGFBP3-probe, 6FAM–5′–***T***CG G***T***T G***T***T ***T***AG GGC GAA GTA CGG G–3′–BHQ-1 (GenBank No. M35878, nucleotides 1760–1784) [38]. Other primers and probes were previously described [24]. The PCR condition was as follows: initial denaturation at 95°C for 10 minutes followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. A standard curve was made for each PCR plate by duplicated PCR amplifications for *COL2A1* on bisulfite-converted human genomic DNA at four different concentrations (in a 5-fold dilution series). The percentage of methylated reference (PMR; i.e., degree of methylation) at a specific locus was calculated by dividing the *GENE/COL2A1* ratio of template amounts in a sample by the *GENE/COL2A1* ratio of template amounts in *Sss*I-treated human genomic DNA (presumably fully methylated) and multiplying this value by 100 [37]. A PMR cutoff value of 4 (except for 6 in *CRABP1* and *IGF2*) was based on previously validated data [36]. Precision and performance characteristics of bisulfite conversion and subsequent MethyLight assays have been previously evaluated and the assays have been validated [36]. CIMP-high was defined as the presence of $\geq$ 6 of 8 methylated promoters, CIMP-low as 1 to 5 of 8 methylated promoters, and CIMP-0 as the absence (0 of 8) of methylated promoters, according to the previously established criteria [23].

Tissue Microarrays (TMAs) and Immunohistochemistry for p53 and p21 (CDKN1A)

Tissue microarrays were constructed as previously described [39]. TMAs were constructed using the Automated Arrayer (Beecher Instruments, Sun Prairie, WI). Briefly, two 0.6-mm tissue cores each from a tumor and normal colonic mucosa were placed in each TMA block. Each TMA block will have a total of approximately 400 cores (100 cases). We examined two to four tumor tissue cores for each marker. A previous validation study have shown that examining two TMA cores can yield comparable results to examining whole tissue sections in more than 95% of cases [40]. We examined whole tissue sections for p21 in all cases, and for p53 in cases for which no tissue block was available for TMAs or results were equivocal in TMAs. Immunohistochemistry for p53 and p21 was performed as previously described [41,42]. p53 positivity was defined as 50% or more of tumor cells with unequivocal strong nuclear staining, as this high threshold has been shown to improve specificity [43]. p21 loss was defined as less than 5% of tumor cells with nuclear staining. Appropriate positive and negative controls were included in each run of immunohistochemistry. All immunohistochemically stained slides were interpreted by one of the investigators (S.O.) blinded from any other clinical and laboratory data.

Statistical Analysis

In the statistical analysis, chi-square test (or Fisher's exact test when the number in any category was less than 10) was performed for categorical data, and kappa coefficients were calculated to determine the degree of agreement between two observers, using SAS program (Version 9.1, SAS Institute, Cary, NC). All P values were two-sided and statistical significance was set at $P \leq .05$.

Results

IGFBP3 Promoter Methylation Is Correlated with CIMP-High

Using MethyLight technology, we quantified DNA methylation in *IGFBP3* and a panel of eight promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) [23,24]. The latter eight promoters constitute a sensitive and specific marker panel for CIMP [23]. Among the 902 tumors, 258 (29%) were positive for *IGFBP3* promoter methylation. There was no significant difference in the frequencies of *IGFBP3* methylation between men (28%) and women (30%). Sensitivity and specificity of *IGFBP3* methylation for the diagnosis of CIMP-high ($\geq$ 6 of 8 methylated promoters, not including *IGFBP3*) were 60% and 77%, respectively (Table 1). Thus, *IGFBP3* methylation was not an excellent marker for CIMP-high, but was still positively correlated with CIMP-high ($P < .0001$). Because 5/8 methylated tumors showed borderline features between

Table 1. Sensitivity and Specificity of *IGFBP3* Methylation for the Diagnosis of CIMP-High.

		CIMP-High (≥ 6/8 Methylated Promoters)	Non–CIMP-High (≤5/8 Methylated Promoters)	Total
IGFBP3 Methylation	Positive	79 (sensitivity 60%*)	179 (23%)	258
	Negative	53 (40%)	591 (specificity 77%†)	644
Total		132	770	902

*Sensitivity is defined as the number of *IGFBP3*-positive CIMP-high cases divided by the number of all CIMP-high cases.
†Specificity is defined as the number of *IGFBP3*-negative non–CIMP-high cases divided by the number of all non–CIMP-high cases.
CIMP, CpG island methylator phenotype.

CIMP-high and CIMP-low [23], we excluded those tumors from further analyses.

We also quantified *IGFBP3* methylation in normal colon tissue in four *IGFBP3*-methylated tumor cases and 12 *IGFBP3*-unmethylated tumor cases. Only one normal sample among the four *IGFBP3*-methylated tumor cases showed *IGFBP3* methylation, and all of the other normal samples showed unmethylated *IGFBP3*.

Inverse Relationship between IGFBP3 and MSI in CIMP-High Tumors

Because molecular classification based on MSI and CIMP status is increasingly important [26], we stratified tumors into four categories according to MSI and CIMP status (Figure 1). Within CIMP-high tumors, the frequency of *IGFBP3* methylation was significantly higher in MSI-L/MSS CIMP-high tumors (85%) than MSI-H CIMP-high tumors (49%, $P < .0001$), indicating an inverse relationship between *IGFBP3* methylation and MSI in CIMP-high tumors. CIMP-low/0 tumors showed low frequencies (17–22%) of *IGFBP3* methylation regardless of MSI status.

IGFBP3 Methylation, CIMP, and Pathologic Features

Because *IGFBP3* methylation is positively correlated with CIMP-high, we stratified tumors according to *IGFBP3* and CIMP status in subsequent analyses (as in Tables 2 and 3). Then, we could examine the effect of *IGFBP3* methylation on various pathologic and molecular features independent of CIMP status.

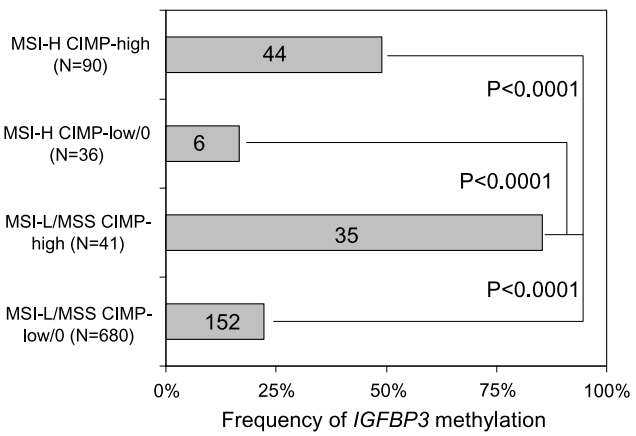

Figure 1. *Frequency of* IGFBP3 *methylation in four MSI/CIMP subtypes of colorectal cancer.* CIMP, *CpG island methylator phenotype;* MSI, *microsatellite instability;* MSS, *microsatellite stable.*

Table 2 summarizes the relations between *IGFBP3* methylation and pathologic features in colorectal cancer. Proximal tumor location, poor differentiation, mucinous features, tumor-infiltrating lymphocytes, and Crohn's-like reaction were significantly associated with *IGFBP3* methylation in all cases, but no significant correlations persisted after tumors were stratified by CIMP status. These findings indicate that those features are associated primarily with CIMP, but not directly with *IGFBP3* methylation.

IGFBP3 Methylation, CIMP, and Other Molecular Features

Table 3 summarizes the relations between *IGFBP3* methylation and other molecular features in colorectal cancer. Whereas MSI and *IGFBP3* were not significantly correlated in all cases and CIMP-low/0 cases, MSI and *IGFBP3* methylation were inversely correlated in CIMP-high tumors ($P < .0001$).

Interestingly, *IGFBP3* methylation and *TGFBR2* mutation were inversely correlated ($P = .02$) in MSI-H CIMP-low/0 tumors, and *IGFBP3* methylation and *BAX* mutation were positively correlated ($P = .02$) in MSI-H CIMP-high tumors (Table 3). These results may suggest possible interactions between the pathways related to these molecules (Figure 2).

BRAF mutation, 18q LOH, and p21 loss were correlated with *IGFBP3* methylation in all cases, but the relations did not persist after tumors were stratified by CIMP status.

Relationship between IGFBP3 Methylation and MSI according to p53 or p21 Status

Because IGFBP3 is one of the downstream effectors of the p53 pathway (Figure 2), we examined the interrelationship between MSI, *IGFBP3*, and p53 (Figure 3). The inverse relationship between MSI and *IGFBP3* methylation (in CIMP-high tumors) persisted in p53-negative tumors ($P < .0001$), but not in p53-positive tumors. These results suggest that *IGFBP3* methylation may be more important in *TP53* wild-type tumors than in *TP53*-mutated tumors, which may have already downregulated IGFBP3.

We also examined the interrelationship between MSI, *IGFBP3*, and p21, one of the downstream effectors of p53 (Figure 4). The inverse relationship between MSI and *IGFBP3* methylation persisted regardless of p21 status, suggesting that p21 and IGFBP3 functions were not directly linked.

Discussion

We conducted this study to examine *IGFBP3* methylation in colorectal cancer, particularly in relation to MSI, CIMP, and p53. Molecular classification of colorectal cancer according to MSI and CIMP is increasingly important [26], because MSI

Table 2. Frequencies of Specific Clinical and Pathologic Features in Colorectal Cancer according to *IGFBP3* Methylation and CIMP Status.

Clinical and Pathologic Features	*N*	All Cases		*P*	CIMP-High		*P*	CIMP-Low/0		*P*
		IGFBP3 Methylation			*IGFBP3* Methylation			*IGFBP3* Methylation		
		(+)	(−)		(+)	(−)		(+)	(−)	
All Cases	902	259	643		80	53		161	582	
Men	405	112	293		29	14		74	275	
Women	497	147	350		51	39		87	307	
Tumor Location										
Total Examined	522	148	374		48	33		87	334	
Proximal	248	97 (66%)	151 (40%)	< .0001	44 (92%)	31 (94%)		42 (48%)	117 (35%)	.02
Distal	274	51 (34%)	223 (60%)		4 (8.3%)	2 (6.1%)		45 (52%)	217 (65%)	
Tumor Differentiation										
Total Examined	885	255	630		80	53		158	568	
Well/Moderate	802	221 (87%)	581 (92%)	.01	57 (71%)	36 (68%)		148 (94%)	538 (95%)	
Poor	83	34 (13%)	49 (7.8%)		23 (29%)	17 (32%)		10 (6.3%)	30 (5.3%)	
Mucinous/Signet Ring Cell Features										
Total Examined	782	228	554		76	49		136	497	
Nonmucinous Carcinoma	471	100 (44%)	371 (67%)	< .0001	26 (34%)	20 (41%)		69 (51%)	346 (70%)	< .0001
Mucinous										
1–100%	311	128 (56%)	183 (33%)		50 (66%)	29 (59%)		67 (49%)	151 (30%)	
1–49%	191	78 (34%)	113 (20%)		24 (32%)	12 (24%)		45 (33%)	100 (20%)	
≥ 50%	120	50 (22%)	70 (13%)		26 (34%)	17 (35%)		22 (16%)	51 (10%)	
Nonsignet Ring Cell Carcinoma	721	207 (91%)	514 (93%)		62 (82%)	39 (80%)		130 (96%)	468 (94%)	
Signet Ring Cells										
1–100%	61	21 (9.2%)	40 (7.2%)		14 (18%)	10 (20%)		6 (4.4%)	29 (5.8%)	
1–49%	46	17 (7.5%)	29 (5.2%)		12 (16%)	9 (18%)		4 (2.9%)	19 (3.8%)	
≥ 50%	15	4 (1.8%)	11 (2.0%)		2 (2.6%)	1 (2.0%)		2 (1.5%)	10 (2.0%)	
Tumor-Infiltrating Lymphocytes										
Total Examined	877	251	626		79	51		155	566	
Absent/Mild	778	210 (84%)	568 (91%)	.003	50 (63%)	39 (57%)		145 (94%)	533 (94%)	
Moderate/Severe	99	41 (16%)	58 (9.3%)		29 (37%)	22 (43%)		10 (6.5%)	33 (5.8%)	
Crohn's-Like Reaction										
Total Examined	674	198	476		58	41		124	428	
Absent/Mild	611	172 (87%)	439 (92%)	.03	38 (66%)	28 (68%)		120 (97%)	404 (94%)	
Moderate/Severe	63	26 (13%)	37 (7.7%)		20 (34%)	13 (32%)		4 (3.2%)	24 (5.6%)	
Peritumoral Lymphocytic Reaction										
Total Examined	879	275	604		76	51		182	541	
Absent/Mild	782	241 (88%)	541 (90%)		47 (63%)	39 (57%)		172 (95%)	493 (91%)	
Moderate/Severe	97	34 (12%)	63 (10%)		29 (37%)	22 (43%)		10 (5.5%)	48 (8.9%)	
Tumor Border										
Total Examined	726	219	507		69	43		136	455	
Circumscribed	262	74 (34%)	188 (37%)		26 (38%)	16 (37%)		45 (33%)	167 (37%)	
Infiltrative	464	145 (66%)	319 (63%)		43 (62%)	27 (63%)		91 (67%)	288 (63%)	
Extraglandular Necrosis										
Total Examined	896	259	637		80	52		161	576	
< 20%	802	230 (89%)	572 (90%)		64 (80%)	37 (71%)		149 (93%)	526 (91%)	
≥ 20%	94	29 (11%)	65 (10%)		16 (20%)	15 (29%)		12 (7.5%)	50 (8.7%)	

Only significant *P* values are described.
CIMP, CpG island methylator phenotype.

and CIMP represent global genomic and epigenomic phenomena, respectively, in tumor cells, and largely determine clinical, pathologic, and molecular features of colorectal cancer. We have found that *IGFBP3* methylation is inversely associated with MSI in CIMP-high tumors, but not in CIMP-low/0 tumors, and that this inverse relationship is limited to p53-negative tumors, but not p53-positive tumors. Our findings imply the complex interrelationship between genomic/epigenomic phenomena (such as MSI and CIMP) and single molecular events, *IGFBP3* methylation and p53 alteration, in colorectal cancer.

Transcriptional inactivation of tumor suppressor genes by promoter CpG island methylation is an important mechanism in human carcinogenesis [14]. Epigenetic aberrations have been reported in various tumor-related genes [14,44–47]. For quantitative DNA methylation analysis, we used MethyLight, which is robust and can reproducibly differentiate low-level methylation from high-level methylation [36,48,49]. Our resource of a large number of colorectal cancers, derived from two large prospective cohorts (relatively unbiased samples compared to retrospective or single-hospital–based samples), has enabled us to precisely estimate the frequency of colorectal cancers with a specific molecular feature (e.g., *IGFBP3* methylation, MSI-H, and so forth). The large number of samples has also provided a sufficient power to accurately estimate the frequency of *IGFBP3* methylation in rare tumor subtypes, such as MSI-L/MSS CIMP-high, CIMP-high p53-positive, and so forth.

IGFBP3 is one of the important downstream effectors of the p53 pathway [8,10], and is also known to interact with the TGF-β pathway [11–13] (Figure 2). Thus, we have examined the interrelationship between *IGFBP3* methylation, p53 positivity, and MSI, and found that the inverse correlation between *IGFBP3* methylation and MSI in CIMP-high tumors

Table 3. Frequencies of Specific Molecular Features in Colorectal Cancer according to *IGFBP3* Methylation and CIMP Status.

Molecular Features	*N*	All Cases		*P*	CIMP-High		*P*	CIMP-Low/0		*P*
		IGFBP3 Methylation			*IGFBP3* Methylation			*IGFBP3* Methylation		
		(+)	(−)		(+)	(−)		(+)	(−)	
MSI										
Total Examined	874	255	619		79	52		158	558	
MSI-H	127	50 (20%)	77 (12%)		44 (56%)	46 (88%)	< .0001	6 (3.8%)	30 (5.4%)	
MSI-L/MSS	747	205 (80%)	542 (88%)		35 (44%)	6 (12%)		152 (96%)	528 (95%)	
KRAS										
Total Examined	860	250	610		77	51		155	550	
Mutant	313	102 (41%)	211 (35%)		14 (18%)	4 (7.8%)		79 (51%)	204 (37%)	.002
Wild-Type	547	148 (59%)	399 (65%)		53 (82%)	47 (92%)		76 (49%)	346 (63%)	
BRAF										
Total Examined	860	250	610		77	51		155	550	
Mutant	112	59 (24%)	53 (8.7%)	< .0001	45 (58%)	32 (63%)		8 (5.2%)	18 (3.3%)	
Wild-Type	748	191 (76%)	557 (91%)		32 (42%)	19 (37%)		147 (95%)	532 (97%)	
18q LOH										
Total Examined (Only Non−MSI-H Tumors)	540	151	389		29	5		107	377	
(+)	353	85 (56%)	268 (69%)	.006	18 (62%)	2 (40%)		63 (59%)	261 (69%)	.04
(−)	187	66 (44%)	121 (31%)		11 (38%)	3 (60%)		44 (41%)	116 (31%)	
p53*										
Total Examined	891	257	634		80	53		159	573	
(+)	386	109 (42%)	277 (44%)		21 (26%)	9 (17%)		81 (51%)	263 (46%)	
(−)	505	148 (58%)	357 (56%)		59 (74%)	44 (83%)		78 (49%)	310 (54%)	
p21*										
Total Examined	864	251	613		79	50		155	555	
Loss	508	124 (49%)	384 (63%)	.0003	22 (28%)	9 (18%)		92 (59%)	371 (67%)	
(+)	356	127 (51%)	229 (37%)		57 (72%)	41 (82%)		63 (41%)	184 (33%)	
TGFBR2 Mutation										
Total Examined (Only MSI-H Tumors)	127	50	77		44	46		6	30	
(+)	94	37 (74%)	57 (74%)		37 (84%)	39 (85%)		0	18 (60%)	.02
(−)	33	13 (26%)	20 (26%)		7 (16%)	7 (15%)		6 (100%)	12 (40%)	
BAX Mutation										
Total Examined (Only MSI-H Tumors)	126	50	76		44	45		6	30	
(+)	32	18 (36%)	14 (18%)	.03	18 (41%)	8 (18%)	.02	0	5 (17%)	
(−)	94	32 (64%)	62 (82%)		26 (59%)	37 (82%)		6 (100%)	25 (83%)	

*p53 and p21 status was determined by immunohistochemistry.
Only significant *P* values are described.
CIMP, CpG island methylator phenotype; *LOH*, loss of heterozygosity; *MSI*, microsatellite instability.

is limited to p53-negative tumors. Thus, *IGFBP3* methylation may be important in *TP53* wild-type tumors, whereas *TP53*-mutated tumors may have already downregulated IGFBP3 and *IGFBP3* methylation may be less relevant. We have also found an inverse correlation between *IGFBP3* methylation and *TGFBR2* mutation in MSI-H CIMP-low/0 tumors, as well as a positive correlation between *IGFBP3* methylation and *BAX* mutation in MSI-H CIMP-high tumors. These complex correlations suggest the intricate relationship between global genomic/epigenomic phenomena (such as MSI and CIMP), these single molecular events (such as *IGFBP3* methylation, *TP53* mutation, *TGFBR2* mutation, and so forth), and the related pathways in colorectal cancer.

In summary, *IGFBP3* promoter methylation in colorectal cancer is inversely associated with MSI in CIMP-high colo-

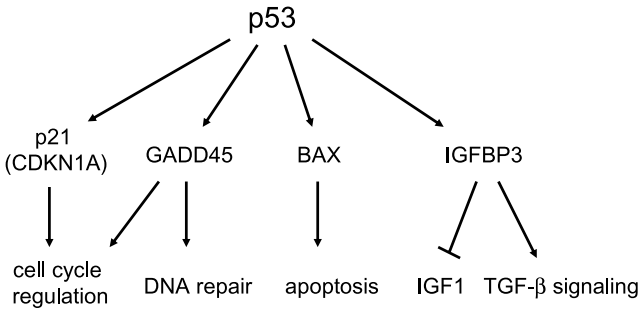

Figure 2. *The p53 pathway and* IGFBP3.

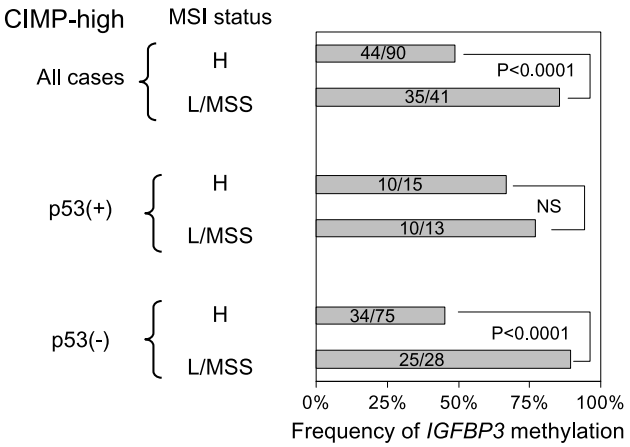

Figure 3. *Frequency of* IGFBP3 *methylation in CIMP-high tumors according to p53 and MSI status. Note that the inverse relationship between MSI and* IGFBP3 *methylation is present in p53-negative tumors, but not in p53-positive tumors.* CIMP, *CpG island methylator phenotype;* MSI, *microsatellite instability;* MSS, *microsatellite stable;* NS, *not significant.*

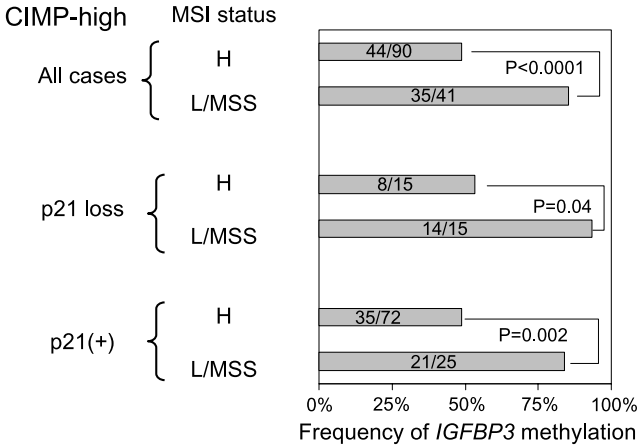

Figure 4. *Frequency of* IGFBP3 *methylation in CIMP-high tumors according to p21 and MSI status. Note that the inverse relationship between MSI and* IGFBP3 *methylation is present regardless of p21 status.* CIMP*, CpG island methylator phenotype;* MSI*, microsatellite instability;* MSS*, microsatellite stable.*

rectal cancer, and this inverse correlation is limited to p53-negative tumors. Further studies are necessary to elucidate the exact pathogenic role of *IGFBP3* promoter methylation in colorectal cancer.

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