Table 3. Clinicopathological Features of the Colorectal Tumors Analyzed Using Array CGH

Feature	C-cluster 1 (n = 49)	C-cluster 2 (n = 13)	C-cluster 3 (n = 22)	P value
Age (years)	69.51 ± 11.01	69.54 ± 8.97	67 ± 10.55	
Sex				
F	14 (28.57)	9 (69.23)	4 (18.18)	
M	35 (71.43)	4 (30.77)	18 (81.82)	
Location				
Right	27 (55.1)	9 (69.23)	6 (27.27)	
Left	8 (16.33)	4 (30.77)	7 (31.82)	
Rectum	14 (28.57)	0	9 (40.91)	
Precursor lesions				
HP	1 (2.04)	0	0	
SSA	2 (4.08)	0	0	
TSA	7 (14.29)	1 (7.69)	0	
Tubular adenoma	3 (6.12)	1 (7.69)	0	
Tubulovillous adenoma	1 (2.04)	0	1 (4.55)	
CIS	0	0	2 (9.09)	
CRCs	9 (18.37)	2 (15.38)	8 (36.36)	
SSA + CIS				
SSA portion	2 (4.08)	0	0	
CIS portion	1 (2.04)	1 (7.69)	0	
Tubulovillous adenoma + CIS				
Tubulovillous adenoma portion	12 (24.49)	1 (7.69)	0	
CIS portion	4 (8.16)	6 (46.15)	3 (13.64)	
Tubular adenoma + CIS				
Tubular adenoma portion	5 (10.2)	0	3 (13.64)	
CIS portion	2 (4.08)	1 (7.69)	5 (22.73)	
KRAS				
Mut	22 (45)	11 (85)	10 (45)	
Wt	27 (55)	2 (15)	12 (55)	
BRAF				
Mut	10 (20)	1 (8)	0	
Wt	39 (80)	12 (92)	22 (100)	
CIMP				
High	17 (35)	4 (31)	0	<0.01
Low	11 (22)	4 (31)	1 (5)	
Negative	21 (43)	5 (38)	21 (95)	

F, female; M, male; Mut, mutated; Wt, wild type.

We performed quantitative bisulfite pyrosequencing of the 25 markers in a total of 330 specimens consisting of 192 precursor lesions, 38 CISs, and 100 CRCs (Table 2). Consistent with the MCAM results, unsupervised hierarchical clustering using the pyrosequencing data revealed that, in addition to malignant lesions (CISs and CRCs), precursor lesions could also be divided into three relative subclasses (M-clusters 1 to 3; Figure 1B). The *BRAF* mutation was significantly enriched in M-cluster 1 tumors, in which most of the genes were highly methylated, suggesting that this subclass corresponded to CIMP-H (Figure 1B). M-cluster 2 included tumors with intermediate levels of methylation and a prevalent *KRAS* mutation, which corresponded to CIMP-L, whereas M-cluster 3 tumors exhibited the lowest methylation levels, which corresponded to CIMP-N. Among the precursor lesions, 37 (19.3%) exhibited a *BRAF* mutation, and most of those [24 (64.9%) of 37] were categorized as CIMP-H (Figure 1C). In CISs and CRCs, the *KRAS* mutation was most enriched in the CIMP-L group, but this tendency was less apparent among the precursor lesions (Figure 1C).

We next used principle component analysis to further evaluate our bisulfite pyrosequencing results and found that the first and second components accounted for 53.1% of the total variance (Figure 1D; see also Supplemental Table S4 at *http://ajp.amjpathol.org*). Two-dimensional plotting then showed that the characteristic pattern for each M-cluster was shared by the precursor and malignant lesions (Figure 1D; see also Supplemental Figure S4A at *http://ajp.amjpathol.org*).

Our hierarchical clustering analysis also showed that the marker genes could be categorized into three subgroups (Figure 1B). Group A genes, which were methylated in most of the samples, corresponded to the type A (age-related) genes originally proposed by Toyota et al.[7] Group B and C genes appeared to correspond to type C (cancer-specific) genes, whereas group C genes in this study were more strongly associated with CIMP-H. Interestingly, among *BRAF*-mutant precursor lesions, adenomas showed much higher levels of methylation of groups B and C genes than HPs (Figure 2). Similar results were obtained with *KRAS*-mutant precursors, although the difference was not statistically significant (Figure 2). By contrast, methylation was minimally up-regulated in adenomas in which both *BRAF* and *KRAS* were wild type, suggesting that accumulation of aberrant methylation, in concert with a *BRAF* or a *KRAS* mutation, may promote the progression from benign tumors to precancerous lesions.

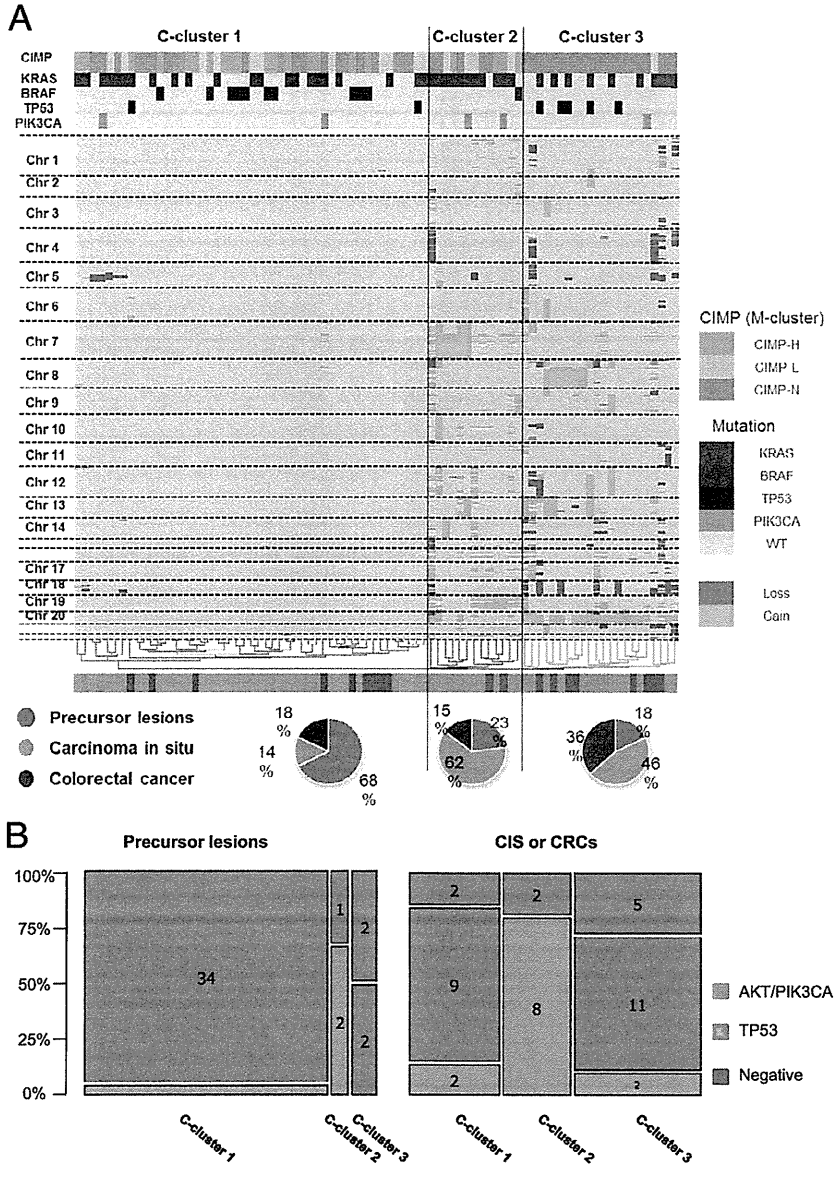

Figure 3. Distinct subclasses of precursor and malignant colorectal lesions are defined based on their CNAs. **A:** Unsupervised hierarchical clustering analysis using array CGH data from 40 precursor lesions, 25 CISs, and 19 CRCs. Lesions could be categorized into three subclasses (C-clusters 1 to 3). CIMP status and gene mutations are indicated (**top panel**), as are chromosome (Chr) numbers (**left panel**). Ratios of precursor lesions, CISs, and CRCs in each C-cluster are shown (**bottom panel**). **B:** Ratios of genetic defects in *AKT/PIK3CA* pathway genes and *TP53* mutations in precursor (**left panel**) and advanced (**right panel**) lesions with the indicated CNA status.

Clinicopathological Features of CIMP-Positive Precursor Lesions

The associations between the clinicopathological characteristics and the CIMP status of the precursor lesions, CISs, and CRCs are summarized in Table 2. Age, sex, and tumor location were matched among the three groups. As with CRCs, CIMP-positive precursor lesions were more prevalent than CIMP-N precursor lesions among female and older patients. Interestingly, CIMP-L was associated with larger diameters among precursor lesions, although this tendency was not apparent among malignant lesions. Most of the CIMP-H precursor lesions were SSAs, whereas most of the CIMP-L precursor lesions were tubulovillous adenomas (Table 2; see also Supplemental Figure S4B at *http://ajp.amjpathol.org*). Levels of groups B and C gene methylation were higher in SSAs than in the other precancerous lesions, whereas methylation of group A genes was higher in all precursor types than in normal colonic tissue (see Supplemental Figure S4C at *http://ajp.amjpathol.org*).

CNAs Are Late Events in Colorectal Tumorigenesis

Several studies have shown an inverse relationship between CIMP and CIN in CRC.[17,33,34] For that reason, we next used array CGH to analyze CNAs in 40 precursor lesions, 25 CISs, and 19 CRCs (Table 3). Unsupervised hierarchical clustering analysis using the array CGH data revealed that the tumors could be categorized into three subclasses, according to their CNA status (C-clusters 1 to 3): C-cluster 1 was enriched in tumors with the fewest

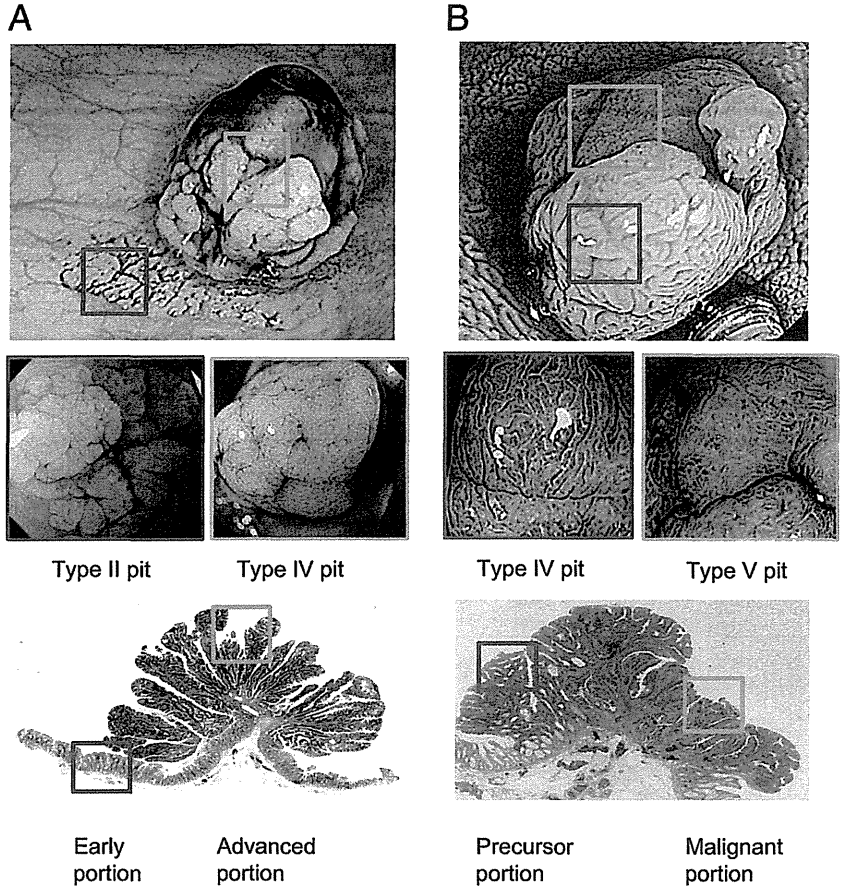

Figure 4. Endoscopic and histological findings in a set of mixed colorectal lesions. **A:** Endoscopic and histological findings from a representative precursor lesion in which a flat portion with an early pit pattern (type II, early portion) is present, along with a protruding portion with advanced pits (type IV, advanced portion). Both components are histologically premalignant (HP and TSA). Biopsy specimens were obtained from the respective portions (**blue** and **red boxes**), after which the molecular profiles are analyzed. **B:** Endoscopic and histological findings from a representative lesion in which a precursor portion (type IV, pit pattern) is present, along with a malignant portion (type V, pit pattern). Biopsy specimens were obtained from the respective portions (**blue** and **red boxes**), after which the molecular profiles are analyzed.

CNAs, whereas C-cluster 2 tumors were characterized by frequent copy number gains on chromosomes 7 and 19; both gains and losses were prevalent among tumors in C-cluster 3 (Figure 3A; see also Supplemental Figure S5A at *http://ajp.amjpathol.org*). Much of the precursor lesions [33 (82.5%) of 40] were enriched in C-cluster 1, whereas most of the malignant lesions (CISs and CRCs) were enriched in C-cluster 2 or 3, suggesting that CNAs occurred late during colorectal tumorigenesis (Figure 3A). Most of the CIMP-positive precursors and malignant lesions were categorized as C-cluster 1 or 2 (Figure 3A; see also Supplemental Figure S6A at *http://ajp.amjpathol.org*), and the methylation levels of the groups B and C genes were similar between C-cluster 1 and 2 tumors (see Supplemental Figure S6B at *http://ajp.amjpathol.org*). These observations suggested that CIMP and CNAs were inversely correlated in many colorectal tumors and that a subset of the CIMP-positive tumors (C-cluster 2) exhibited frequent copy number gains, particularly on chromosomes 7 and 19. As was previously seen, we found that most *BRAF*-mutant precursors and malignant lesions were enriched in C-cluster 1 (Figure 3A). By contrast, although most of the *KRAS*-mutant precursors were enriched in C-cluster 1, *KRAS*-mutant malignant lesions were equally distributed among all three C-clusters (Figure 3A; see also Supplemental Figure S6C at *http://ajp.amjpathol.org*).

The results of our integrative genetic and epigenetic analysis of precursor lesions were indicative of several distinct molecular pathways leading to CRC development. Notably, CIMP-positive/*BRAF*-mutant CRCs did not exhibit more CNAs than did pre-invasive lesions with the same *BRAF* mutations and CIMP-positive methylation profile, suggesting that such pre-invasive lesions may progress to CRC without additional CNAs. By contrast, CIMP-positive/*KRAS*-mutant precursors appeared to develop via CNA-independent and CNA-dependent pathways. The CNA-dependent pathway was characterized by frequent amplification of *BRAF* and *EZH2* on chromosome 7q and amplification of *AKT2*/*PAK4* and *DNMT1* on chromosome 19 (see Supplemental Figure S5B at *http://ajp.amjpathol.org*). More important, we found that most C-cluster 2 tumors exhibited genetic defects (mutations and/or CNAs) in genes whose products were implicated in the AKT/PIK3CA pathway, including *AKT1*, *AKT2*/*PAK4*, *PDK1*, and *PIK3CA* (Figure 3B; see also Supplemental Figure S5B at *http://ajp.amjpathol.org*).

Dynamics of the Molecular Signatures during the Progression of Colorectal Tumorigenesis

Our results suggested that acquisition of CNAs was essential for *BRAF* wild-type precursors to progress to more

Table 4. Histological and Molecular Signatures in a Set of Mixed Colorectal Lesions

Precursor Lesion Plus Precursor Lesion									
Type II or III pit patterns (early potion)					Type IV pit pattern (advanced potion)				
Pathological findings	Mutation	CIMP (M-cluster)	C-cluster	MSI	Pathological findings	Mutation	CIMP (M-cluster)	C-cluster	MSI
SSA	*BRAF*	CIMP-H	ND	Negative	Adenoma	*BRAF*	CIMP-H	ND	Negative
SSA	*BRAF*	CIMP-H	ND	Negative	Adenoma	*BRAF*	CIMP-H	ND	Negative
SSA	*KRAS*	CIMP-H	ND	Negative	SSA	*KRAS*	CIMP-H	ND	Negative
SSA	*KRAS*	CIMP-H	ND	Negative	Adenoma	*KRAS*	CIMP-H	ND	Negative
SSA	*BRAF*	CIMP-H	1	Negative	Adenoma	*BRAF*	CIMP-H	1	Negative
SSA	*KRAS*	CIMP-L	ND	Negative	SSA	*KRAS*	CIMP-H	ND	Negative
HP	*BRAF*	CIMP-H	ND	Negative	SSA	*BRAF*	CIMP-H	ND	Negative
HP	*BRAF*	CIMP-H	1	Negative	SSA	*BRAF*	CIMP-H	1	Negative
TSA	*KRAS*	CIMP-L	1	Negative	TSA	*KRAS*	CIMP-H	2	Negative
TSA	*KRAS*	CIMP-L	1	Negative	TSA	*KRAS*	CIMP-L	1	Negative
TSA	*KRAS*	CIMP-N	ND	Negative	TSA	*KRAS*	CIMP-L	ND	Negative
HP	*BRAF*	CIMP-N	ND	Negative	TSA	*BRAF*	CIMP-H	ND	Negative
HP	*BRAF*	CIMP-N	1	Negative	TSA	*BRAF*	CIMP-H	1	Negative
HP	*BRAF*	CIMP-N	ND	Negative	TSA	*BRAF*	CIMP-H	ND	Negative
HP	WT	CIMP-N	ND	Negative	TSA	WT	CIMP-L	ND	Negative
HP	*BRAF*	CIMP-N	ND	Negative	TSA	*BRAF*	CIMP-L	ND	Negative
Tubular adenoma	*KRAS*	CIMP-N	1	Negative	Tubulovillous adenoma	*KRAS*	CIMP-H	1	Negative
Tubulovillous adenoma	WT	CIMP-L	ND	Negative	Tubulovillous adenoma	WT	CIMP-L	ND	Negative
Tubulovillous adenoma	WT	CIMP-L	ND	Negative	Tubulovillous adenoma	WT	CIMP-L	ND	Negative
Tubular adenoma	*KRAS*	CIMP-N	2	Negative	Tubulovillous adenoma	*KRAS*	CIMP-N	3	Negative
Tubular adenoma	WT	CIMP-N	1	Negative	Tubular adenoma	*KRAS*	CIMP-N	1	Negative
Tubular adenoma	WT	CIMP-N	1	Negative	Tubular adenoma	*KRAS*	CIMP-N	1	Negative

Precursor Lesion Plus CIS or CRC									
Type II, III, or IV pit patterns (precursor potion)					Type V pit pattern (malignant potion)				
Pathological findings	Mutation	CIMP (M-cluster)	C-cluster	MSI	Pathological findings	Mutation	CIMP (M-cluster)	C-cluster	MSI
SSA	*BRAF*	CIMP-H	1	Negative	CIS	*BRAF*	CIMP-H	1	Positive
SSA	*BRAF*	CIMP-H	ND	Negative	CIS	*BRAF*	CIMP-H	ND	Positive
SSA	*BRAF*	CIMP-H	1	Negative	CIS	*BRAF*	CIMP-H	2	Positive
Tubular adenoma	*KRAS*	CIMP-H	1	Negative	CIS	*KRAS*	CIMP-H	1	Negative
Tubulovillous adenoma	*KRAS*	CIMP-H	1	Negative	CIS	*KRAS*	CIMP-H	1	Negative
Tubulovillous adenoma	*KRAS*	CIMP-L	ND	Negative	CIS	*BRAF*, *TP53*	CIMP-H	ND	Positive
Tubulovillous adenoma	*KRAS*	CIMP-L	1	Negative	CIS	*KRAS*	CIMP-H	2	Negative
Tubulovillous adenoma	*KRAS*	CIMP-L	1	Negative	CIS	*KRAS*	CIMP-L	2	Negative
Tubulovillous adenoma	WT	CIMP-N	1	Negative	CIS	*KRAS*	CIMP-L	2	Negative
Tubulovillous adenoma	*KRAS*	CIMP-N	2	Negative	CIS	*KRAS*	CIMP-L	2	Negative
Tubular adenoma	*KRAS*	CIMP-N	1	Negative	CIS	*KRAS*, *PIK3CA*	CIMP-L	2	Negative
Tubulovillous adenoma	*KRAS*	CIMP-N	1	Negative	CIS	*KRAS*	CIMP-N	2	Negative
Tubulovillous adenoma	*PIK3CA*	CIMP-N	1	Negative	CRC	*PIK3CA*	CIMP-N	2	Negative
Tubulovillous adenoma	WT	CIMP-N	1	Negative	CIS	*KRAS*	CIMP-N	1	Negative
Tubulovillous adenoma	WT	CIMP-L	ND	Negative	CIS	*KRAS*	CIMP-L	ND	Negative
Tubular adenoma	WT	CIMP-N	ND	Negative	CIS	WT	CIMP-L	ND	Negative
Tubulovillous adenoma	*KRAS*	CIMP-L	1	Negative	CIS	*KRAS*, *TP53*	CIMP-L	1	Negative
Tubulovillous adenoma	*KRAS*	CIMP-N	1	Negative	CRC	*KRAS*	CIMP-N	3	Negative
Tubulovillous adenoma	WT	CIMP-N	1	Negative	CIS	WT	CIMP-N	3	Negative
Tubulovillous adenoma	WT	CIMP-N	1	Negative	CIS	*KRAS*	CIMP-N	3	Negative
Tubular adenoma	WT	CIMP-N	1	Negative	CIS	WT	CIMP-N	3	Negative
Tubular adenoma	*KRAS*	CIMP-L	1	Negative	CRC	*KRAS*, *TP53*	CIMP-L	3	Negative
Tubular adenoma	WT	CIMP-N	3	Negative	CIS	WT	CIMP-N	3	Negative
Tubular adenoma	*TP53*	CIMP-N	3	Negative	CIS	*TP53*	CIMP-N	3	Negative
Tubular adenoma	WT	CIMP-N	3	Negative	CIS	WT	CIMP-N	3	Negative
Tubular adenoma	WT	CIMP-N	1	Negative	CIS	WT	CIMP-N	1	Negative
Tubulovillous adenoma	WT	CIMP-N	1	Negative	CIS	WT	CIMP-N	1	Negative

ND, no data; WT, wild type.

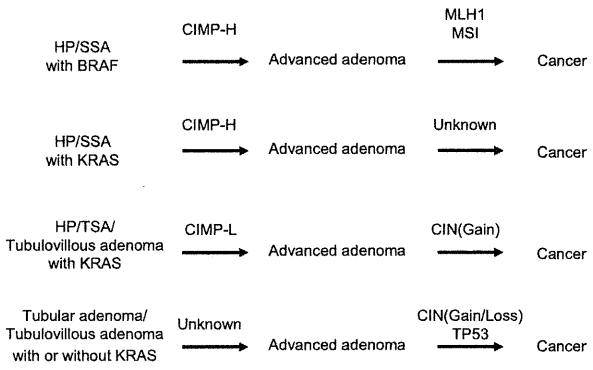

Figure 5. Model for development of CRCs via four distinct molecular pathways.

advanced tumors. To confirm this finding, we analyzed a series of colorectal lesions in which precursor components were present together with more advanced lesions within the same tumors. According to Kudo's classification, the aberrant pit patterns observed using magnifying colonoscopy are hallmarks of malignant tumors, and enabled us to distinguish between the precursor and advanced components (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).[19] We first analyzed the precursor lesions (n = 22), in which portions with early pit patterns (type II or III) were present, along with more advanced pits (type IV), although both components were histologically premalignant (Figure 4A). Progression from precursor lesions with early pits to lesions with advanced pits was associated with the accumulation of DNA methylation, whereas genetic alterations (mutations and CNAs) were rarely acquired (Table 4; see also Supplemental Figures S7 and S8 at *http://ajp.amjpathol.org*). By contrast, progression from precursor (type II, III, or IV pit) to malignant (type V pit) lesions (n = 27) was accompanied by the occurrence of a wide variety of genetic changes, whereas methylation levels remained largely unchanged (Figure 4B and Table 4; see also Supplemental Figures S7 and S8 at *http://ajp.amjpathol.org*). For example, CIMP-H adenomas with a *BRAF* mutation acquired MSI as they developed into CISs, suggesting that inactivation of *MLH1* and subsequent genetic instability were late events in the CIMP-H pathway. In addition, CIMP-L and CIMP-N adenomas acquired mutations and CNAs as they developed into CISs and CRCs. Most malignant lesions that exhibited C-cluster 2–type CNAs were derived from tubulovillous adenomas and were characterized by a *KRAS* mutation and CIMP-L (Table 4). On the other hand, most advanced lesions with C-cluster 3–type CNAs were CIMP negative, and more than half of those lesions were derived from tubular adenomas (Table 4).

Discussion

In the present study, we performed integrated genetic and epigenetic analyses with many colorectal neoplasias, including premalignant and malignant lesions. Because of the tight association between CIMP and the clinicopathological features of CRCs, it was anticipated that epigenetic profiling of premalignant lesions would provide important information that would aid in selecting appropriate therapeutic options and predicting clinical outcomes.[35–37] Numerous studies have confirmed that CRCs arise through the accumulation of both genetic and epigenetic alterations; however, the interactions between these alterations early during carcinogenesis remained largely uninvestigated. In addition, only a small fraction of colorectal adenomas may develop into malignant tumors, and progression from adenoma to cancer generally takes >10 years.[38] Thus, the identification of genetic and/or epigenetic alterations that directly correlate with the malignant potential of precursor lesions could facilitate risk assessment and enable prevention of CRCs.

Our comprehensive methylation analysis revealed that the aberrant methylation patterns characterizing CIMP are established early during colorectal tumorigenesis. We found that CIMP-H precursor lesions are strongly associated with SSA and *BRAF*/*KRAS* mutations, whereas CIMP-L precursor lesions are associated with tubulovillous adenomas and frequent *KRAS* mutations. Recently, Yagi et al[39] reported that colorectal adenomas could be classified into high-, intermediate-, and low-methylation epigenotypes, and that the intermediate-methylation epigenotype correlated significantly with a *KRAS* mutation, which is consistent with our observations. These results are indicative of the important relationship between the histological type and the molecular features of premalignant colorectal lesions. By contrast, CIMP-N precursor lesions contained various histological types, including HP, tubular adenoma, and tubulovillous adenoma. Recent reports have shown that a subset of HPs with *BRAF* or *KRAS* mutations progress to SSAs or TSAs, but the time at which aberrant methylation occurs remains unclear.[15,40] Our present findings indicate that, among *KRAS*- or *BRAF*-mutant precursors, the methylation levels of several genes were significantly increased during the progression from HP to adenoma. Concurrent increases in the methylation of multiple genes were further confirmed in mixed lesions containing HP and adenoma components (Table 4).

CIN is an important driving force promoting colorectal tumorigenesis, and recent studies have shown an inverse relationship between CIMP and CIN.[17,33,34,41] Our genome-wide CNA analysis of precursor and malignant lesions revealed that most CNAs are acquired during the progression from adenomas to CISs/CRCs. Consistent with earlier reports, CIMP-H tumors showed few CNAs, whereas both copy number gains and losses were prevalent among CIMP-N tumors.[17,33,34] Furthermore, we discovered that a subset of *KRAS*-mutant/CIMP-L tumors exhibited a unique CNA pattern characterized by frequent copy number gains at chromosomes 7 and 19, with relatively few copy number losses.

In contrast to the tight association between a *BRAF* mutation and CIMP-H in a subset of CRCs, the relationship between a *KRAS* mutation and CIMP status is not fully understood, which likely reflects the molecular complexity of *KRAS*-mutant CRCs.[10,12,13,42] Our analysis sug-

gests that *KRAS*-mutant precursors progress to CRCs via three distinct pathways (Figure 5). First, such as *BRAF*-mutant/CIMP-H tumors, a subset of *KRAS*-mutant tumors, derived from SSAs, exhibit high levels of methylation and few CNAs. Although methylation of *MLH1* is essential for *BRAF*-mutant/CIMP-H adenomas to develop into cancers, *MLH1* methylation was infrequent among *KRAS*-mutant/CIMP-positive tumors. SSAs with a *KRAS* mutation are presumed to be the origin of MSI-negative/CIMP-H CRCs, although the molecular mechanisms underlying the progression from precursors to malignant lesions remain unknown.

Second, we identified a subclass of *KRAS*-mutant/CIMP-positive cancers originating from tubulovillous adenomas or TSAs, in which alteration in *AKT/PIK3CA* signaling was crucially involved. In these tumors, genes associated with an *AKT/PIK3CA* signaling pathway were commonly affected during the progression from adenomas to malignant lesions. Several studies have shown aberrant *AKT/PIK3CA* signaling to be critical for CRC development, and mutations in *PIK3CA*, *AKT1*, *AKT2*, and *PDK1* and amplification of *AKT2/PAK4* are all reportedly associated with a poor prognosis.[43–46] In the present study, concurrent amplification of *AKT2/PAK4* was specifically observed in *KRAS*-mutant/CIMP-positive cancers. Interestingly, we also observed frequent amplification of *BRAF* in this type of tumor. Although further study is needed to clarify its functional role in tumorigenesis, recent studies have shown that *BRAF* amplification promotes acquired resistance to MAPK/ERK kinase 1/2 inhibitors in CRC cells.[47,48]

The molecular profiles of mixed precursor lesions suggest that CIMP is acquired during the progression from flat to protruding-type adenomas (Figure 4A; see also Supplemental Figures S2D, S7A, and S8A at *http://ajp.amjpathol.org*), whereas the *KRAS* mutation status was unchanged between the two components. This means that a *KRAS* mutation precedes CIMP in the *KRAS*/CIMP pathway, and that the acquisition of epigenetic changes, in addition to the *KRAS* mutation, may promote adenoma cell proliferation.

By contrast, *KRAS*-mutant/CIMP-N tumors are derived from tubular adenomas or tubulovillous adenomas and are characterized by a frequent *TP53* mutation and high levels of CNA. An analysis of CIMP-N mixed lesions revealed that a *KRAS* mutation is found only in some advanced components, suggesting that a *KRAS* mutation occurs late during tumorigenesis in this pathway. Thus, *KRAS* mutations appear to play multiple roles during colorectal carcinogenesis. Further study will be required to fully characterize the functional diversity of *KRAS* mutations in CRCs; however, based on the genetic and epigenetic alterations found in CRC and the timing of the occurrence of *KRAS* mutations, we propose that CRCs develop via the four distinct pathways illustrated in Figure 5.

The results of several recent studies support the two-colon concept, which suggests that MSI-positive, CIMP-positive, and *BRAF*-mutant CRCs occur more frequently in the proximal colon.[9–11,13] By contrast, Yamauchi et al[49,50] recently reported that the frequencies of CIMP-H, MSI-high, and *BRAF* mutations in CRCs increased gradually along colorectal subsites from the rectum to the ascending colon. Because of the relatively few samples, we could not confirm this continuum concept in our present study. However, given its potentially significant impact on both basic and clinical research in CRC, testing this theory through further study of a larger population would seem warranted.

Our findings in this study have important implications for translating the molecular basis of carcinogenesis into a clinical benefit. The detection of high-risk precursor lesions is essential for preventing CRCs, and pit pattern observation using magnifying endoscopy enables us to detect neoplastic lesions with malignant potential.[19] We have dissected the morphological, histological, and molecular alterations in precancerous lesions of the colorectum and determined that they are directly linked to one another. Moreover, we provide strong evidence that aberrant pit patterns reflect histological changes and genetic and epigenetic defects in the precursor lesions, and that intratumoral variation in pit patterns could be predictive of the extent of the molecular abnormalities in a given tumor. Such a microstructure-based diagnostic system is readily available to the clinician, although specific skills are required for detailed pit pattern analysis. Further advances in the algorithm for pattern recognition may lead to the development of an innovative diagnostic system for detecting premalignant lesions and a reduction in CRC mortality.

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Review Article

Model of translational cancer research in multiple myeloma

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Recently, intensive laboratory and preclinical studies have identified and validated therapeutic molecular targets in multiple myeloma (MM). The introduction of novel agents such as the proteasome inhibitor bortezomib and the immunomodulatory drugs thalidomide and lenalidomide, which were rapidly translated from preclinical studies at the Dana-Farber Cancer Institute into clinical trials, has changed the treatment paradigm and markedly extended overall survival; MM has therefore become a remarkable example of translational cancer research in new drug development. In this article, with the aim of determining the key factors underlying success in translational research, we focus on our studies of MM at Dana-Farber Cancer Institute as well as at our institutes. The identification of these key factors will help to promote translational cancer research not only in MM but also in other hematologic malignancies and solid tumors, to develop novel therapies, to overcome drug resistance, and to thereby improve the prognosis of cancer patients. (*Cancer Sci* 2012; 103: 1907–1912)

Current approaches in multiple myeloma

Multiple myeloma (MM) is a neoplastic plasma cell disorder that is characterized by the clonal proliferation of malignant plasma cells in the bone marrow (BM), the presence of monoclonal immunoglobulin in the serum and/or urine in most cases, and associated organ dysfunction, including lytic bone lesions, compromised immunity, anemia, renal failure, and hypercalcemia.[1] The combined use of melphalan and prednisone since the 1960s provided a median survival of 2–3 years for patients with MM. High-dose melphalan with autologous stem cell transplantation was established in the 1990s and this combination further increased the patient median survival to 3–4 years. However, MM was largely incurable, and therefore, novel biological treatment approaches were urgently required. In the last decade, the introduction of novel agents such as the proteasome inhibitor bortezomib and the immunomodulatory drugs (IMiDs) thalidomide and lenalidomide, which were rapidly translated from preclinical studies into clinical trials carried out by Anderson *et al.* at the Dana-Farber Cancer Institute (DFCI) of Harvard Medical School (Boston, MA, USA), has changed the treatment paradigm and markedly extended the overall survival.[1–5] Multiple myeloma has therefore become a remarkable example of translational cancer research in new drug development.[2] In this review, with the aim of determining the key factors underlying the success of translational research, we focused the several dozen studies we were engaged in at DFCI as well as our institutes.[2,3,6–8] This review consists of the following parts: (i) bases for translational research in oncology; (ii) novel targets and drugs; and (iii) biomarkers.

Bases for translational research in oncology

Research in medical science is traditionally divided into two categories, basic and clinical research. Clinical research, as defined by the US National Institutes of Health (NIH), includes: patient-oriented research carried out on human subjects in which an investigator directly interacts with human subjects; epidemiological and behavioral studies; and research on outcomes and health services.[9] In contrast, basic research is carried out without considering practical ends and provides general knowledge.[9] To improve the prognosis of cancer patients, including those afflicted with MM, basic research and clinical research must be translated into practical applications. Translational research is a term that is used to describe the process by which the results of research carried out in the laboratory, in individuals (clinical), or in populations are used to develop new methods of diagnosis and treatment of a disease (clinical practice). The Translational Research Working Group of the National Cancer Institute (USA) also stated that the goal of translational research in oncology is to transform scientific discoveries arising from laboratory, clinical, or population studies into clinical applications to reduce cancer incidence, morbidity, and mortality.[10] Bridging the gap between basic research and clinical practice is a key factor for effective translational research. The NIH also concluded that barriers between clinical and basic research render translation of new knowledge to the clinic and back again to the laboratory bench difficult.[11] Therefore, translational research requires a close collaboration between basic scientists and clinical researchers, as well as between academia and industry.[2] Other key factors underlying the success of translational research include an understanding of the process of oncogenesis and the disease status as well as the identification of biological indicators for diagnosis, prognosis, and stratification.[12] In MM, the elucidation of tumor biology through clinical observations and genomic analyses accompanying the introduction of particular targeted drugs provide an example of bedside-back-to-bench research.[2] Ongoing translational research in MM includes genetic and epigenetic studies to evaluate myelomagenesis, identify targeted hallmarks of MM, and identify biomarkers to develop improved classification and personalized medicine;

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translational research also includes the development of novel therapies that target MM cells in the BM microenvironment.

Novel targets and drugs

In 2011, Hanahan and Weinberg updated their proposition of the hallmarks of cancer that enable tumor cell growth and progression.[13] The proposed 10 hallmarks listed in Table 1 provide a framework for understanding cancer biology and therapeutic targets, and offer an effective way to organize and describe MM biology and therapeutic targets. For example, because nuclear factor κB (NFκB) activation in MM cells results in proliferative signaling and resistance to cell death, targeting the NFκB pathway is a promising therapeutic strategy in MM.[3,14] The concept of specific molecular targeting has been applied to the development of cancer therapies, and the two main approaches discussed here are the use of small-molecule agents and the use of therapeutic mAbs.[7] In Table 1, we list candidate small-molecule compounds that target proposed hallmarks in MM, most of which we and our colleagues have studied at DFCI and our institutes.[14–33] These small-molecule compounds that interfere with certain hallmarks of cancer are under development and are being investigated in clinical trials; in some cases, they have been approved for clinical use in the treatment of cancer, including MM.[2,8]

In addition, mAb-based therapies for MM are currently being developed.[7,8,34] Figure 1 presents a list of therapeutic antibodies in MM, which can be divided into three classes based on their mechanisms of action.[35] Class I antibodies recognize and bind to cell-bound antigens to kill target cells through crystallizable fragment-mediated effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity, and antibody-dependent apoptosis.[36] The anti-CD38 mAb daratumumab[37] and anti-CD40 mAbs lucatumumab and dacetuzumab[38] are currently in preclinical and clinical development for the treatment of MM.[7,8,34] Our studies show that IMiDs can augment ADCC triggered by mAbs including elotuzumab,[30,36] and this finding provides the rationale for a combination clinical trial.

Class II antibodies also recognize cell-bound antigens, but their proposed mechanism of action does not involve crystallizable fragment effector functions. The main functions of this class of antibodies are to block ligand–receptor interactions and to act as immunoconjugates to convey intracellular toxins or radioactive isotopes. In MM research, novel mAb–maytansinoid immunoconjugates (huN901-DM1, which binds to CD56,[39] and BT062, which binds to CD138[40]) are being developed.

Class III antibodies bind to and neutralize soluble antigens, and their mechanism of action often involves blocking of the soluble ligand from binding to its receptor. A human mAb 1339 targeting interleukin-6 (IL-6) is being developed for MM.[41] The targeting of soluble ligands that affect bone biology by using mAbs such as the anti-RANKL mAb denosumab[42] and the anti-DKK1 mAb BHQ880[43] is promising, not only for preserving bone integrity but also for treating MM.[34] In January 2012, denosumab was approved in Japan for the treatment of bone complications caused by MM.[42]

The biological diversity of tumor cells within the BM microenvironment may influence the number of targets for MM therapies.[2,3,6,8] Candidate agents and approved novel drugs can show significant antitumor activity in MM *in vitro*, but treatment with single agents may not provide sufficient clinical efficacy because of drug resistance. Successful treatments can thus be achieved using other hallmarks and by addressing drug resistance. Therefore, we envisage that the use of functionally multitargeting drugs, as listed in Table 2, will provide effective MM therapies.[2,8]

Proteasome inhibitors. The ubiquitin proteasome pathway regulates the turnover of many intracellular proteins that are tagged with multiple ubiquitin molecules for transport to the 26S proteasome for subsequent degradation. Bortezomib is a prototype 26S proteasome inhibitor that selectively binds to and reversibly inhibits chymotrypsin-like and caspase-like activity.[28,44] In 2001, Hideshima *et al.* reported that bortezomib regulates cell cycle proteins in MM cells and targets intrinsic and extrinsic apoptotic pathways. It also inhibits the secretion of IL-6 and vascular endothelial growth factor

Table 1. Candidate small-molecule compounds targeting hallmarks in multiple myeloma

Hallmark	Candidate agent	Description	References
Sustainment of proliferative signaling	Perifosine	Inhibition of Akt	15
	Adaphostin	Abl cleavage	16
	CAL-101	Inhibition of PI3Kδ	17
	PKF115-584	Inhibition of β-catenin/TCF pathway	18
	SDX-308	Inhibition of β-catenin/TCF pathway	19
Evasion of growth suppressors	Seliciclib	Inhibition of cyclin-dependent kinase	20
Activation of invasion and metastasis	MLN3897	Inhibition of CCR1	21
Enabling replicative immortality	Imetelstat	Inhibition of telomerase	22
Induction of angiogenesis	Pazopanib	Inhibition of VEGFR	23
	IMiDs	Inhibtion of VEGF secretion	24
	Bortezomib	Inhibtion of VEGF secretion	25
Resistance to cell death	ABT-737	Inhibition of Bcl-2/Bcl-XL/Bcl-w	26
	R-etodolac	Upregulation of proapoptotic Mcl-1s	27
	MLN120B	Inhibition of IKKβ	14
	Bortezomib	Inhibition of NFκB	3,29
	IMiDs	Inhibtion of cytokine secretion	3,29
Prevention of immune destruction	IMiDs	Inhibtion of IL-2 secretion	30,60
Deregulation of cellular energetics	Cerulenin	Inhibition of fatty-acid synthase	31
Genome instability and mutation	ABT-888	Inhibition of PARP	32
Tumor-promoting inflammation	MLN120B	Inhibition of IKKβ	14
	BIRB 796	Inhibition of p38 MAPK	33
	IMiDs	Inhibtion of cytokine secretion	3,29

CCR1, chemokine (c-c motif) receptor-1; IKKβ, IKB kinase β; IL-2, interleukin-2; IMiDs, immunomodulatory drugs; NFκB, nuclear factor κB; PARP, poly(ADP-ribose) polymerase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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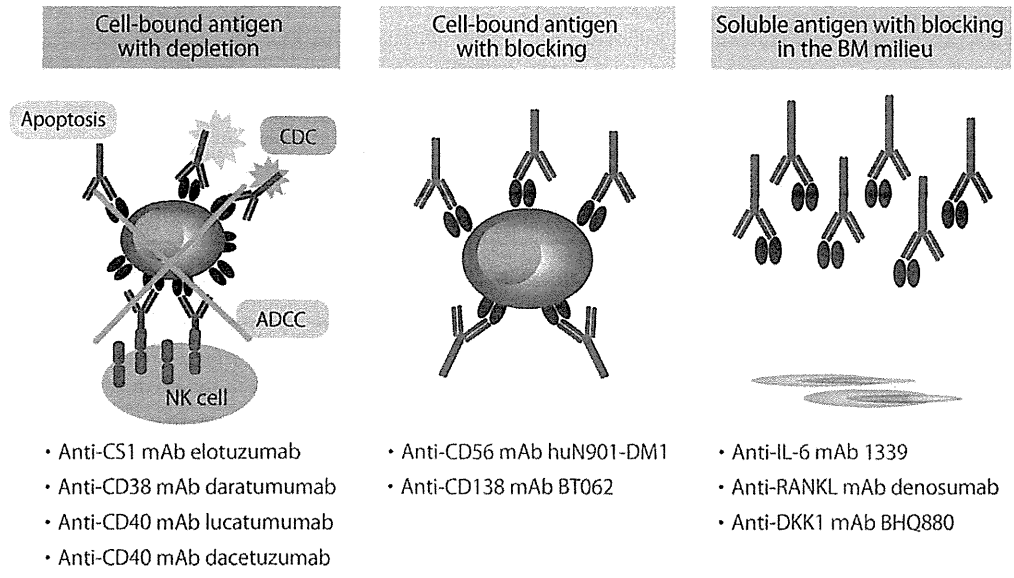

Fig. 1. Classification of therapeutic antibodies based on their mechanisms of action. Class I antibodies recognize and bind to cell-bound antigens. The Fc effector functions are part of the mechanism of action of antibodies. Class II antibodies also recognize and bind to cell-bound antigens but their proposed mechanism of action does not involve Fc effector functions. Class III antibodies bind to and neutralize soluble antigens, and their mechanism of action often involves blocking the soluble ligand from binding to its receptor. ADCC, antibody-dependent cell-mediated cytotoxicity; BM, bone marrow; CDC, complement-dependent cytotoxicity; NK, natural killer.

Table 2. Functionally multitargeting agents in multiple myeloma

Classification	Candidate agent	Description	References
Proteasome inhibitor	Bortezomib	Inhibition of chymotryptic-like and caspase-like activities	28,44
	MLN9708	Inhibition of chymotryptic-like and caspase-like activities	46
	Carfilzomib	Inhibition of chymotryptic-like activity	47
	Marizomib (NPI-0052)	Inhibition of chymotryptic-like, trypsin-like, and caspase-like activities	48
HDAC inhibitor	Vorinostat (SAHA)	Pan-HDAC inhibitor	49
	Panobinostat (LBH-589)	Pan-HDAC inhibitor	50
	Tubacin	HDAC6 selective inhibitor	51
	ACY-1215	HDAC6 selective inhibitor	52
HSP inhibitor	IPI-504 (tanespimycin)	Inhibition of hsp90	54
	SNX-2112	Inhibition of hsp90	55
Drugs influencing lysophospholipid signaling	Perifosine	Inhibition of Akt	15
	FTY720	Sphingosine 1-phosphate agonist	57
	LPAATβ inhibitor	Inhibition of LPAATβ	58
IMiD	Thalidomide	Inhibtion of cytokine secretion	29,59
	Lenalidomide (CC-5013)	Inhibtion of cytokine secretion; induction of apoptosis	29
	Pomalidomide (CC-4047)	Inhibtion of cytokine secretion; induction of apoptosis	29,59
DNA methyltransferase inhibitor	Decitabine	DNA methyltransferase inhibitor	69

HDAC, histone deacetylase; HSP, heat shock protein; IMiDs, immunomodulatory drugs; LPAATβ, lysophosphatidic acid acyltransferase β.

triggered by the binding of MM cells to BM stromal cells and inhibits BM angiogenesis by exerting a direct inhibitory effect on endothelial cells.[25] Bortezomib has undergone a remarkable transition from bench to bedside; a phase II study of bortezomib revealed a 35% response rate with manageable toxicity, and bortezomib was then approved by the US Food and Drug Administration (FDA) for the treatment of relapsed/refractory MM in 2003.[45]

Recently, the orally active agent MLN9708,[46] carfilzomib which selectively inhibits chymotrypsin-like activity,[47] and the broad-based proteasome inhibitor marizomib[48] have been developed in preclinical and clinical studies. Marizomib inhibits chymotrypsin-like, trypsin-like, and caspase-like activity and induces apoptosis in MM cells resistant to conventional agents and bortezomib.[48]

Histone deacetylase inhibitors. Histone deacetylases (HDACs) are enzymes involved in the remodeling of chromatin and play a key role in the epigenetic regulation of gene expression, which ultimately mediates cellular differentiation and survival.[44] The combination of bortezomib with HDAC inhibitors has yielded promising results in preclinical MM models and will thus be applied to clinical trials.

The HDAC inhibitors can be divided into two groups: non-selective pan-HDAC inhibitors such as vorinostat[49] and

panobinostat[50] that predominately target Class I (HDAC1, HDAC2, and HDAC3) and Class IIb (HDAC6) HDACs; and Class I HDAC inhibitors such as romidepsin and entinostat that target only Class I HDACs.[44] Although the mechanism underlying the synergistic activity of HDAC inhibitors with bortezomib is not fully understood, it may involve the role played by HDAC6 in the aggresomal degradation of ubiquitinated proteins.[44,51] The preclinical activity of a novel HDAC6 inhibitor, ACY-1215, alone and in combination with bortezomib, was recently reported[52] and transformed into a clinical study.

Heat-shock protein inhibitors. Heat-shock proteins (HSPs) constitute a class of molecular chaperones that, under normal conditions, facilitate protein folding and regulate the turnover of proteins involved in cell growth and survival. Under conditions of environmental stress, HSP expression increases as an adaptive means to maintain cell homeostasis and enhance cell survival. Because bortezomib induces the expression of stress response-related proteins such as hsp27, hsp70, and hsp90, these proteins are molecular targets for overcoming bortezomib resistance.[53] Inhibition of p38MAPK, which is an upstream molecule of hsp27, enhances the cytotoxicity of bortezomib in MM cells, thereby providing evidence that hsp27 confers bortezomib resistance.[33] Hsp90 inhibitors such as 17-AAG (tanespimycin),[53] IPI-504 (retaspimycin hydrochloride, which is a water-soluble analog of tanespimycin),[54] and SNX-2112[55] enhance bortezomib-induced cytotoxicity in preclinical models. IPI-504 has been translated into a clinical study in MM.[56]

Drugs influencing lysophospholipid signaling. We evaluated several drugs that influence lysophospholipid signaling, such as the sphingosine 1-phosphate analogue FTY720,[57] an LPAATβ inhibitor,[58] and perifosine.[15] Perifosine, which is an alkyl-phosphocholine compound, has been shown to inhibit Akt activation without affecting the activity of PI3K or phosphoinositide-dependent kinase 1. Because perifosine inhibits the Akt activation triggered by bortezomib to enhance MM cytotoxicity *in vitro*, combined therapy with bortezomib and drugs that inhibit Akt signaling is promising. Perifosine in combination with bortezomib is being evaluated in clinical trials.

Immunomodulatory drugs. The IMiDs have several anti-MM effects, including direct cytotoxicity, inhibition of angiogenesis, and induction of tumor immunity, and provide a remarkable example of translational cancer research in MM. In 2000, Hideshima *et al.*[29] reported the mechanism of anti-MM activity of the IMiDs lenalidomide (IMiD3, CC5013) and pomalidomide (IMiD1, CC4047), which potently induce apoptosis or growth arrest in MM cells. The IMiDs also reduce the secretion of IL-6 and vascular endothelial growth factor triggered by the binding of MM cells to BM stromal cells, and they inhibit angiogenesis.[59] Lenalidomide was rapidly applied to clinical trials and was approved by the FDA in 2006 for use in patients who have received prior therapy.[1] The IMiDs also stimulate a T cell co-stimulatory mechanism to induce IL-2 expression and T-cell proliferation.[60] Moreover, IMiDs induce natural killer (NK) cell-mediated cytotoxicity because the proliferation and ADCC of NK cells are induced by IL-2 production.[30] These data provide the cellular and molecular basis for the use of IMiDs as an adjuvant in immunotherapeutic treatment strategies for MM.

Biomarkers

A biomarker, as defined by NIH, is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers can be classified based on their application, such as diagnostic biomarkers, biomarkers for the staging of diseases, biomarkers for disease prognosis, and biomarkers for monitoring the clinical response to an intervention. Genetic heterogeneity has been indicated in MM, and has important implications for tumor pathogenesis, prognosis, and treatment. Importantly, cytogenetic aberrations, including the non-hyperdiploid, cytogenetically detected chromosomal 13q deletion as well as *t*(4,14), *t*(14,16), 1q gain, and del(17p), as detected by FISH, are indicators of high-risk MM associated with a poor outcome.[1] Novel therapies such as bortezomib can overcome, at least in part, the adverse outcome conferred by these abnormalities.[2] However, there has been much less progress in the development of predictive biomarkers for specific treatments.[12] To identify biomarkers to predict the effect of particular targeted therapies, appropriate clinical trial designs are necessary. Phase I studies are needed to establish that the drug inhibits the targeted pathway in the tumor. Phase II studies are required to obtain data for determining predictive biomarkers that identify patients whose tumors are driven by the inhibition of the target molecule so that therapy-specific diagnostic tests can be developed for phase III trials. Because some novel drugs in development in MM have specific molecular targets, the identification of biomarkers that also define drug sensitivity is a promising therapeutic strategy. Examples include the use of PI3K inhibitors in patients who show PI3K activation and IκB inhibitors in patients who show activation of the NFκB pathway. Efforts to examine patient samples by genetic, cytogenetic, and epigenetic methods are important to identify biomarkers to improve patient classification and, if possible, introduce personalized therapy for MM.[2] In this review, we focus on genetic studies that have recently been facilitated by next-generation sequencing technologies, as well as focus on DNA methylation studies that we are engaged in at our institute.

Genetics in MM. Major tumor-genome sequencing projects have been undertaken to identify the numerous genes mutated in cancer.[61] However, the key steps in oncogenesis in human tumors remain unclear. In MM, genomic studies are currently being carried out for the definition of heterogeneity, new target discovery, and development of personalized therapy. The analysis of somatic mutations by sequencing of the tumor genomes in 38 MM cases revealed that the mutated genes involved in NFκB activation, protein homeostasis, and histone methylation are consistent with MM biology.[62] Moreover, activating mutations of *BRAF* were observed in 4% of patients; this finding has immediate clinical translational implications for the use of BRAF inhibitors. It is important to distinguish the driver mutations from the passenger mutations; a driver mutation is defined as a mutation that is causally implicated in oncogenesis, whereas a passenger mutation is defined as a mutation that has no effect on the fitness of a clone but is present in the same genome with a driver mutation.[61] The existence of several driver mutations in individual cancer is consistent with the hallmarks of cancer.[13]

DNA methylation in MM. DNA methylation, which occurs in cytosine bases located 5′ to a guanine in which the cytosine–guanine pairs are known as CpG or CG dinucleotides, is catalyzed by DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B).[63] Various cancers are characterized by promoter hypermethylation and consequent epigenetic silencing of multiple genes, and this process can be reversed during DNA synthesis, which renders it a potential therapeutic target.[63] The DNA methyltransferase inhibitors azacitidine and decitabine (5-aza-2′-deoxycytidine) have remarkable activity in the treatment of myelodysplastic syndrome (MDS), and both were approved by the FDA for the treatment of patients with MDS.[8] We and others studied DNA methylation in MM and identified certain key genes, including *RAS*, *dexamethasone-*

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Table 3. Genes epigenetically silenced in multiple myeloma

Gene	Chromosomal location	Function	References
CDKN2A ($p16^{INK4A}$)	9p21.3	Inhibition of cyclin-dependent kinase	64
CDKN2B ($p15^{INK4B}$)	9p21.3	Inhibition of cyclin-dependent kinase	64
CHFR	12q24.33	Mitotic checkpoint	65
RASSF1A	3p21.31	Inhibition of Ras signaling	66
DAPK1	9q21.33	Induction of programmed cell death	67
BNIP3	10q26.3	Induction of apoptosis	68
RASD1	17p11.2	Modulation of coregulator activity of NONO	69

induced 1 (*RASD1*), listed in Table 3.[64–69] Interestingly, MM cells that showed methylation of RASD1 were resistant to dexamethasone, and treatment with decitabine restored RASD1 expression and enhanced the cytotoxicity of dexamethasone in tumor cells. The methylation levels of RASD1 in clinical samples were elevated after repeated chemotherapy, including therapy with dexamethasone. The goal of our ongoing studies is to define RASD1 methylation as a predictive indicator of steroid resistance in MM. Our findings suggest that epigenetic gene silencing is involved in MM progression and drug resistance, and DNA methylation can therefore be a potential biomarker for MM. We are also engaging in genome-wide methylation analyses to determine the molecular mechanisms underlying MM, including oncogenesis, drug resistance, and the heterogeneity of genetic, cytogenetic, and epigenetic aberrations, thereby identifying biomarkers in MM.

Perspectives and conclusions

Ongoing translational cancer studies in MM include: genetic and epigenetic studies to evaluate myelomagenesis, identify targeted hallmarks of MM, and develop improved classification and personalized medicine; the development of next-generation novel therapies targeting MM cells in the BM milieu; and the development of rationally based combination therapies.[2,3,8] To date, many preclinical studies have hinted at the myriad of pathways that can be targeted for a synergistic and multitargeted approach. To identify these areas of molecular synergism, close collaboration between basic researchers and clinical staff is critical. These efforts will help to develop novel therapies, overcome drug resistance, and improve the prognosis of patients with MM.

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Disclosure statement

The authors have no conflicts of interest.

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Cancer Prevention Research

Research Article

Aberrant Methylation of *RASGRF1* Is Associated with an Epigenetic Field Defect and Increased Risk of Gastric Cancer

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Abstract

Aberrant DNA methylation is implicated in the epigenetic field defect seen in gastric cancer. Our aim in this study was to identify predictive biomarkers by screening for DNA methylation in noncancerous background gastric mucosa from patients with gastric cancer. Using methylated-CpG island amplification coupled with CpG island microarray (MCAM) analysis, we identified 224 genes that were methylated in the noncancerous gastric mucosa of patients with gastric cancer. Among them, *RASGRF1* methylation was significantly elevated in gastric mucosa from patients with either intestinal or diffuse type gastric cancer, as compared with mucosa from healthy individuals (8.3% vs. 22.4%, $P < 0.001$; 8.3% vs. 19.4%, $P < 0.001$). *RASGRF1* methylation was independent of mucosal atrophy and could be used to distinguish both serum pepsinogen test-positive [sensitivity, 70.0%; specificity, 86.7%; area under the receiver operator characteristic (ROC) curve, AUC, 0.763] and -negative patients with gastric cancer (sensitivity, 72.2%; specificity, 87.0%; AUC, 0.844) from healthy individuals. Ectopic expression of *RASGRF1* suppressed colony formation and Matrigel invasion by gastric cancer cells, suggesting it may be involved in gastric tumorigenesis. Collectively, our data suggest that *RASGRF1* methylation is significantly involved in an epigenetic field defect in the stomach, and that it could be a useful biomarker to identify individuals at high risk for gastric cancer. *Cancer Prev Res; 5(10); 1203–12. ©2012 AACR.*

Introduction

Gastric cancer is a major cause of cancer-related mortality, worldwide. *Helicobacter pylori* (*H. pylori*) plays an important role in gastric carcinogenesis, although the majority of the individuals with *H. pylori* infection do not develop gastric cancer (1). Histologically, gastric cancers are divided into 2 subgroups, intestinal and diffuse, which are thought to develop through separate pathologic pathways (2). Etiologic analysis has shown that individuals with *H. pylori*–related gastritis, severe atrophy, and intestinal metaplasia are at high risk of developing intestinal type gastric cancers, which are often associated with metachronous gastric cancer development. On the other hand, individuals with *H. pylori*–related pangastritis and enlarged-fold gastritis, which are lesions without mucosal atrophy or intestinal metaplasia, are at increased risk of developing diffuse type gastric cancers (3). Surveillance of these high-risk patients using reliable and accurate predictive markers is important for reducing the incidence of gastric cancer and its mortality.

Aberrant DNA methylation is one of the most common molecular alterations found in neoplasias; CpG island hypermethylation is associated with the silencing of tumor suppressor genes and other tumor-related genes, whereas global hypomethylation is thought to induce oncogene activation or chromosomal instability (4). The list of genes aberrantly methylated in gastric cancer is growing and now includes genes involved in cell-cycle regulation, apoptosis, immune function, cell signaling, and tumor invasion and metastasis (3, 5). In addition, aberrant DNA methylation is frequently observed in noncancerous gastric mucosa in *H. pylori*–infected patients, suggesting aberrant DNA methylation is an early step during gastric carcinogenesis (6, 7). We previously showed hypomethylation of LINE-1 repetitive elements and hypermethylation of *CDH1* in enlarged-fold gastritis, which is an indicator of a high risk for diffuse type gastric cancer (8). More recently, we and others reported

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frequent hypermethylation of tumor suppressive miRNA genes in the noncancerous gastric mucosa of patients with gastric cancer (9, 10). Taken together, these results suggest that aberrant methylation in the gastric mucosa could be a useful biomarker for evaluating gastric cancer risk.

Our aim in the present study was to identify predictive biomarkers by screening for DNA methylation in the noncancerous background gastric mucosa in cases of gastric cancer. For this purpose, we carried out high-throughput CpG island methylation profiling in a set of noncancerous gastric mucosa specimens from patients with gastric cancer and from cancer-free individuals. We identified a number of aberrantly methylated genes in the gastric mucosa of the patients with gastric cancer, including *RASGRF1*, which was frequently methylated in cases of both intestinal and diffuse type gastric cancer. We provide evidence that *RASGRF1* is a novel target for epigenetic silencing in gastric cancer, and that its methylation in the gastric mucosa is strongly associated with an elevated risk for both types of gastric cancer.

Materials and Methods

Study population and cell lines

A total of 130 primary gastric cancer specimens were obtained through surgical resection or endoscopic biopsy. Samples of noncancerous gastric mucosa were obtained through endoscopic biopsy from 91 patients with gastric cancer and 69 healthy individuals. From each patient, biopsy specimens of noncancerous gastric mucosa were taken from the gastric body and antrum. *H. pylori* infection was assessed using a rapid urease test, a serum antibody test, or a urea breath test. If any one of these assays was positive, the patients were considered to be *H. pylori*–positive. The updated Sydney system and serum pepsinogen test was used to estimate the degree of gastritis (11). The serum pepsinogen test was carried out by assessing the serum pepsinogen I (PGI) and pepsinogen II (PGII) levels; the criteria for positivity were PGI $\leq$ 70 ng/mL and a PGI/PGII ratio $\leq$3.0 (12). Informed consent was obtained from all patients before the collection of specimens. Approval of this study was obtained from the Institutional Review Board of Akita Red Cross Hospital (Akita, Japan) and Sapporo Medical University (Sapporo, Japan).

Gastric cancer cell lines (MKN7, SH101, SNU1, SNU638, JRST, KatoIII, AZ521, AGS, and NCI-N87) were obtained and cultured as described previously (9, 13). SH101 and HSC43 cells were kindly provided by Dr. Kazuyoshi Yanagihara, Yasuda Women's University (14, 15). In some instances, cells were treated with 2 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC; SIGMA) for 72 hours, replacing the drug and medium every 24 hours. Genomic DNA was extracted using the standard phenol–chloroform procedure. Total RNA was extracted using TRIZOL reagent (Invitrogen), and then treated with a DNA-free Kit (Ambion).

Methylated CpG island amplification coupled with CpG island microarray analysis

Methylated CpG island amplification (MCA) was conducted as described previously (16, 17). Briefly, 500 ng of genomic DNA was digested with the methylation-sensitive restriction endonuclease *SmaI* (New England Biolabs), after which it was digested with the methylation-insensitive restriction endonuclease *XmaI*. The adaptors were prepared by addition of the oligonucleotides RMCA12 (5′-CCGGGCAGAAAG-3′) and RMCA24 (5′-CCACCGCCATCCGAGCCTTTCTGC-3′). After the ligation of the digested DNA to the adaptors, PCR amplification was carried out. Using a BioPrime Plus Array CGH Genomic Labeling System (Invitrogen), MCA amplicons from gastric cancers and samples of *H. pylori*–positive noncancerous gastric mucosa were labeled with Alexa Fluor 647, and those from pooled samples of a mixture of *H. pylori*–negative normal gastric mucosa were labeled with Alexa Fluor 555. Labeled MCA amplicons were then hybridized to a custom human CpG island microarray containing 15,134 probes covering 6,157 unique genes (G4497A; Agilent Technologies; ref. 18). After washing, the array was scanned using an Agilent DNA Microarray Scanner (Agilent Technologies), and the data were processed using Feature Extraction software ver. 10.7 (Agilent Technologies). The data were then analyzed using GeneSpring GX ver. 11 (Agilent Technologies) after which unsupervised hierarchical clustering analysis were carried out using JMP ver. 8 (SAS Institute). The microarray data in this study have been submitted to the Gene Expression Omnibus (GEO) and accession number is GSE39175.

Methylation analysis

Genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (QIAGEN) after which methylation-specific PCR (MSP), bisulfite sequencing, and pyrosequencing were carried out as described previously (9). Briefly, for bisulfite pyrosequencing, the biotinylated PCR product was purified, made single-stranded, and used as a template in a pyrosequencing reaction run according to the manufacturer's instructions. The pyrosequencing reaction was carried out using a PSQ96 system with a PyroGold Reagent Kit (QIAGEN), and the results were analyzed using Q-CpG software (QIAGEN). For bisulfite sequencing, amplified PCR products were cloned into pCR2.1-TOPO vector (Invitrogen), and 12 to 14 clones from each sample were sequenced using an ABI3130x automated sequencer (Applied Biosystems). Primer sequences and PCR product sizes are listed in Supplementary Table S1.

RT-PCR of *RASGRF1*

Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen), after which the integrity of the cDNA was confirmed by amplifying glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primer sequences and PCR product sizes are shown in Supplementary Table S1. Quantitative reverse transcription-PCR (RT-PCR) was carried out using TaqMan Gene Expression Assays (*RASGRF1*, Hs00182314_m1; *GAPDH*, Hs99999905_m1; Applied Biosystems) and a 7500 Fast Real-Time PCR System

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(Applied Biosystems). SDS ver. 1.4 software (Applied Biosystems) was used for comparative ΔC_t analysis.

Construction of a *RASGRF1* expression vector

A full-length *RASGRF1* cDNA was amplified by PCR using cDNA derived from *RASGRF1*-expressing AZ521 cells as a template and then cloned into pcDNA3.2/V5/GW/D-TOPO (Invitrogen). The sequence was then verified. Primer sequences and PCR product sizes are shown in Supplementary Table S1.

Western blot analysis

Western blot analysis was carried out as described previously (9). Mouse anti-V5 monoclonal antibody (mAb; Invitrogen), rabbit anti-RASGRF1 polyclonal Ab (sc-863, Santa Cruz Biotechnology), and mouse antiactin mAb (Chemicon) were used as instructed by the manufacturers. The immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Biosciences).

Colony formation assays

Colony formation assays were carried out as described previously (13). Briefly, cells (1×10^5 cells) were transfected with 4 μg of *RASGRF1* expression vector or empty pcDNA3.1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were then plated on 60-mm culture dishes and selected for 10 days in 0.4 mg/mL G418. Colonies were stained with Giemsa and counted using the NIH IMAGE software.

Matrigel invasion assays

Cell invasion was assessed using Matrigel invasion assays as described previously (19). Gastric cancer cells (2×10^6 cells) were transfected with 5 μg of *RASGRF1* expression vector or a negative control using a Cell Line Nucleofector kit V (Lonza) with a Nucleofector I electroporation device (Lonza) according to the manufacturer's instructions. After incubation for 24 hours, 1×10^5 transfectants suspended in 500 μL of serum-free RPMI-1640 medium were added to the tops of BD BioCoat Matrigel Invasion Chambers (BD Biosciences) prehydrated with PBS, and 700 μL of RPMI-1640 media supplemented with 10% FBS were added to the lower wells of the chambers. After incubation for 22 hours, the invading cells were stained with 1% toluidine and then counted in 3 randomly selected microscopic fields per membrane.

Statistical analysis

To compare differences in continuous variables between groups, *t* tests or ANOVA with *posthoc* Tukey tests were conducted. Fisher exact test or χ^2 test was used for analysis of categorical data. Receiver operator characteristic (ROC) curves were constructed on the basis of the levels of methylation. Values of $P < 0.05$ (2-sided) were considered statistically significant. Statistical analyses were carried out using SPSS statistics 18 (IBM Corporation) and GraphPad Prism ver. 5.0.2 (GraphPad Software).

Results

Identification of *RASGRF1* methylation in background gastric mucosa in gastric cancer

To screen for methylation changes early during gastric carcinogenesis, we compared the methylation status of noncancerous background gastric mucosa from patients with gastric cancer with that in samples of gastric mucosa from healthy individuals. To accomplish this, we carried out methylated-CpG island amplification coupled with CpG island microarray (MCAM) analysis using a set of *H. pylori*–positive gastric mucosa specimens from otherwise healthy individuals (gastric antrum, $n = 11$; gastric body, $n = 15$), noncancerous gastric mucosa from patients with gastric cancer (gastric antrum, $n = 10$; gastric body, $n = 10$), gastric cancer tissues ($n = 10$), and gastric cancer cell lines (AGS, HSC43, KatoIII, MKN74, and NUGC4). The gastric mucosa specimens from the healthy individuals were divided into 2 histologic groups: antrum-predominant gastritis ($n = 14$), which has a low risk for gastric cancer and pangastritis or corpus-predominant gastritis ($n = 12$), which has a high risk for gastric cancer. We thus aimed to identify genes that showed greater methylation in noncancerous antral gastric mucosa from patients with gastric cancer than in mucosa from healthy individuals with antrum-predominant gastritis. MCAM analysis revealed 889 unique genes that were hypermethylated in the background gastric mucosa in intestinal type gastric cancer, as compared with antrum-predominant gastritis (Fig. 1A, Supplementary Table S2). In addition, we identified 478 unique genes that were methylated in the background mucosa in diffuse type gastric cancer (Fig. 1A, Supplementary Table S3). Subsequent Venn diagram analysis identified 224 genes that were methylated in the background gastric mucosae of patients with either type of gastric cancer but not in healthy individuals (Fig. 1A, Supplementary Table S4).

Unsupervised hierarchical clustering analysis using MCAM data for the 224 selected genes revealed that the genes could be categorized into 3 subclasses (Fig. 1B, Supplementary Table S4): group 1 genes (81 unique genes) were methylated in the majority of antral gastric mucosae from patients with gastric cancer and in gastric cancer tissues; group 2 genes (35 unique genes) were prevalently methylated in patients with gastric cancer and in otherwise healthy individuals with pangastritis or corpus-predominant gastritis, suggesting the methylation was inflammation-related and less cancer-specific; and group 3 genes (108 unique genes), which gave an elevated signal in the antrum of patients with gastric cancer and patients with pangastritis or corpus-predominant gastritis, but overall, the levels of methylation were relatively low in all of the specimens tested. These results suggest that genes predictive of gastric cancer risk are likely enriched in group 1. Among the group 1 genes, we selected 11 (*RASGRF1*, *SOX5*, *GALNT14*, *RGS20*, *RPIB9*, *SYT5*, *WNT3*, *BASP1*, *ITGA4*, *KCNV1*, and *PAX5*) that gave the highest microarray signals in the gastric cancer tissues and background gastric mucosa. Using MSP, we tested their methylation status in a small set of clinical specimens and found that 3 genes (*RASGRF1*, *GALNT14*,

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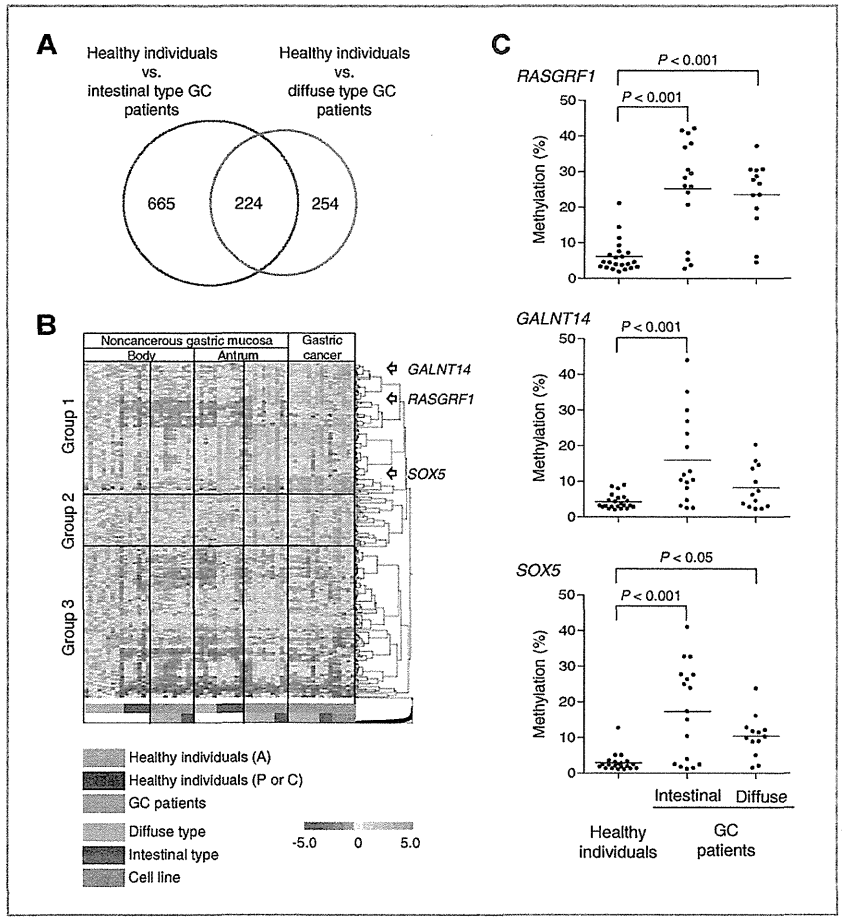

Figure 1. Identification of genes methylated in the background gastric mucosa in gastric cancer. A, MCAM analysis identified 889 and 478 genes that were differentially methylated (>1.5-fold) between antrum-predominant gastritis from otherwise healthy individuals and noncancerous gastric mucosa from patients with intestinal or diffuse type gastric cancer, respectively. Venn diagram analysis revealed 224 genes that were methylated in the background gastric mucosa in both gastric cancer types. B, unsupervised hierarchical clustering analysis of the MCAM data obtained from patients with antrum-predominant gastritis (A), pangastritis, or corpus-predominant gastritis (P or C), noncancerous mucosae from patients with gastric cancer, gastric cancer tissues, and gastric cancer cell lines using the selected 224-gene set. Each row represents a single probe and each column represents a sample. Three genes (*RASGRF1*, *GALNT14*, and *SOX5*) were selected from among the group 1 genes. C, summarized results of bisulfite pyrosequencing of *RASGRF1*, *GALNT14*, and *SOX5* in a set of gastric mucosa specimens from healthy individuals ($n = 22$) and noncancerous gastric mucosae from patients with intestinal type gastric cancer ($n = 16$) or diffuse type gastric cancer ($n = 13$).

and *SOX5*) strongly discriminated between healthy individuals and patients with gastric cancer (Fig. 1B, Supplementary Fig. S1). We therefore used quantitative bisulfite pyrosequencing to assess their methylation levels in a set of antral mucosae from *H. pylori*–positive healthy individuals ($n = 22$) and noncancerous gastric mucosae from the antrum of patients with intestinal ($n = 16$) or diffuse ($n = 13$) type gastric cancer (Fig. 1C, Supplementary Fig. S2). Consistent with the MCAM and MSP data, the levels of methylation of these 3 genes were elevated in the background gastric mucosae from patients with gastric cancer, although methylation of *GALNT14* in diffuse type patients with gastric cancer was less pronounced. In contrast, methylation of *RASGRF1* was significantly elevated in patients with either type of gastric cancer, suggesting it could be a useful biomarker for predicting gastric cancer risk.

Analysis of *RASGRF1* methylation and expression in gastric cancer

On the basis of the results summarized earlier, we selected *RASGRF1* for further analysis. The promoter region and exon 1 of *RASGRF1* are embedded within a typical CpG island (Supplementary Fig. S3). Bisulfite pyrosequencing revealed that *RASGRF1* was highly methylated in a majority of the gastric cancer cell lines tested (Fig. 2A), and that expression of *RASGRF1* mRNA was absent in those cells. Treatment with a DNA methyltransferase inhibitor, 5-aza-dC, restored *RASGRF1* mRNA expression in multiple cell lines, suggesting *RASGRF1* is epigenetically silenced in gastric cancer cells (Fig. 2A). In contrast, methylation levels were low in MKN74 and AZ521 cells, in which *RASGRF1* mRNA was abundantly expressed (Fig. 2A and data not shown). Elevated levels (>15%) of *RASGRF1* methylation were also frequently detected in both types of primary gastric cancer (intestinal type, 31 of 62, 50.0%; diffuse type, 41 of 68, 60.3%; Fig. 2B, Supplementary Table S5). We also found that *RASGRF1* methylation correlates inversely with higher pathologic T (pT) categories and distant metastasis (Supplementary Table S5). When the methylation status of *RASGRF1* was further assessed in selected specimens, we observed that its CpG island is densely methylated in gastric cancer cell lines, primary tumors, and background

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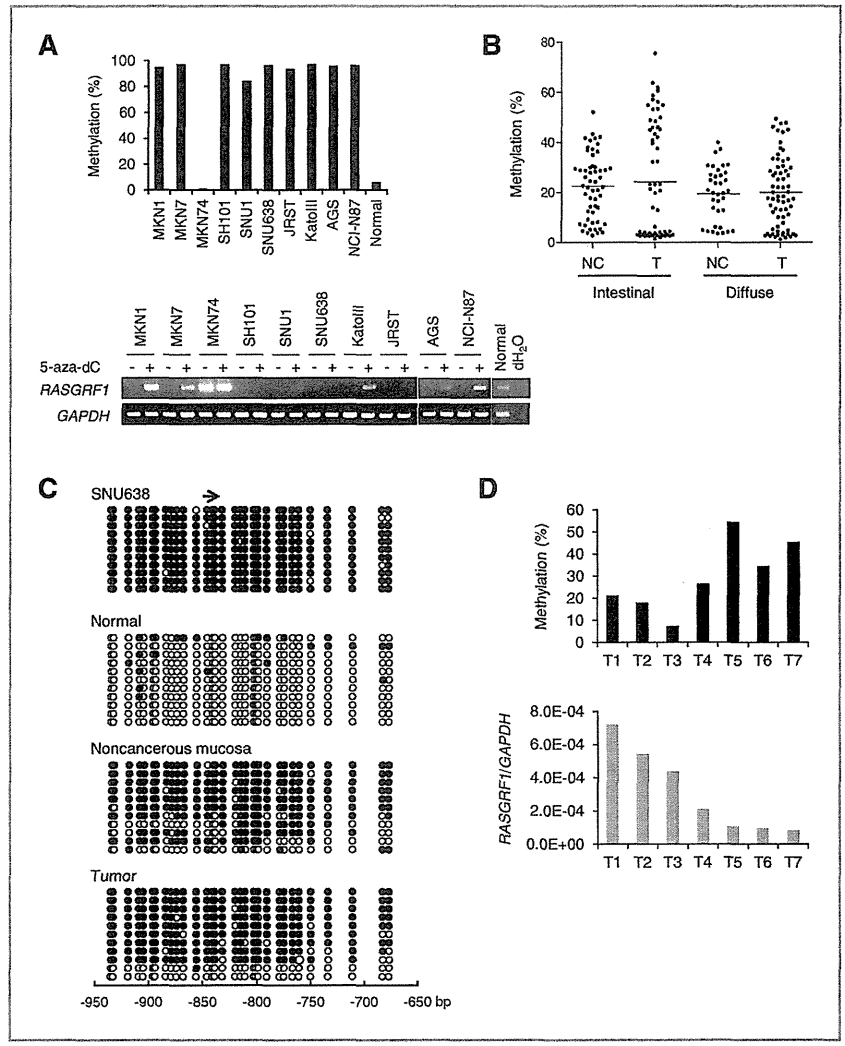

Figure 2. Analysis of *RAGRF1* methylation and expression in gastric cancer. A, bisulfite pyrosequencing of *RASGRF1* in the indicated gastric cancer cell lines and a sample of normal gastric mucosa (top). RT-PCR analysis of *RASGRF1* in gastric cancer cell lines with (+) or without (-) 5-aza-dC treatment, and in normal stomach (bottom). RT-PCR analysis of *GAPDH* was carried out with all samples to ensure the cDNA quality; dH_2O indicates no RNA added. B, summary of bisulfite pyrosequencing in primary tumors (T) from patients with intestinal type gastric cancer ($n = 62$) and diffuse type gastric cancer ($n = 68$). Noncancerous gastric mucosae (NC) from patients with intestinal type gastric cancer ($n = 55$) and diffuse type gastric cancer ($n = 36$) are also shown. C, representative bisulfite sequencing of the *RASGRF1* promoter in a gastric cancer cell line (SNU638), normal gastric mucosa, primary samples of noncancerous gastric mucosa, and a gastric cancer tumor. Open and filled circles represent unmethylated and methylated CpG sites, respectively. The region analyzed by bisulfite pyrosequencing is indicated by an arrow on the top. D, methylation and expression of *RASGRF1* in a set of primary gastric cancer tissues. Methylation levels were determined by bisulfite pyrosequencing (top) and expression was assessed by quantitative RT-PCR (bottom).

noncancerous gastric mucosa, whereas normal gastric mucosa from a healthy individual showed only limited methylation (representative results in Fig. 2C). To test whether methylation of *RASGRF1* is associated with its downregulation in primary gastric cancer, we carried out bisulfite pyrosequencing and quantitative RT-PCR with a set of tissue specimens, which revealed an inverse relationship between methylation and expression (Fig. 2D).

Increased *RASGRF1* methylation in noncancerous gastric mucosae from gastric cancer patients

The elevated levels of *RASGRF1* methylation in the background gastric mucosa of patients with gastric cancer suggest its involvement in an epigenetic field defect. We therefore next assessed its clinical usefulness as a predictive biomarker of gastric cancer risk by comparing the levels of *RASGRF1* methylation in a set of gastric mucosa specimens from healthy individuals (*H. pylori*–negative, $n = 12$; *H. pylori*–positive, $n = 50$) and noncancerous gastric mucosa specimens from patients with gastric cancer (intestinal type, $n = 55$; diffuse type, $n = 36$). All specimens were collected from the gastric antrum, and the clinicopathologic features of the samples are shown in Table 1. In healthy individuals, the levels of *RASGRF1* methylation did not significantly differ between *H. pylori*–negative and -positive gastric mucosae (5.4% and 9.0%, average 8.3%), suggesting that severe *RASGRF1* methylation is not induced by *H. pylori* infection alone (Fig. 3A). In contrast, methylation levels were significantly elevated in noncancerous mucosae from intestinal and diffuse type patients with gastric cancer (22.5% and 19.4%, average 21.3%), indicating that *RASGRF1* methylation in noncancerous

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Table 1. Clinicopathologic features of the patients in this study

		Healthy individuals ($n = 69$)	Patients with cancer		
			Noncancerous mucosa ($n = 91$)	Gastric cancer tissues ($n = 130$)	Total ($n = 290$)
Age	Mean	60.0	70.1	66.7	66.2
	SD	12.8	9.0	11.4	11.7
Sex	Male	50 (72.5%)	65 (71.4%)	87 (66.9%)	
	Female	17 (24.6%)	26 (28.6%)	36 (27.7%)	
	Unknown	2 (2.9%)	0 (0.0%)	7 (5.4%)	
H. pylori	Positive	50 (72.5%)	41 (45%)	24 (18.5%)	115 (39.7%)
	Negative	12 (17.4%)	14 (15.4%)	5 (3.8%)	31 (10.7%)
	Unknown	7 (10.1%)	36 (39.6%)	101 (77.7%)	144 (49.7%)
Histology	Intestinal type		55 (60.4%)	62 (47.7%)	
	Diffuse type		36 (39.4%)	68 (52.3%)	
PG	Positive	15 (21.7%)	20 (22.0%)	9 (6.9%)	44 (15.2%)
	Negative	23 (33.3%)	18 (19.8%)	11 (8.5%)	52 (17.9%)
	Unknown	31 (44.9%)	53 (58.2%)	110 (84.6%)	194 (66.9%)

gastric mucosae may be associated with gastric cancer risk (Fig. 3A).

We also generated a ROC curve to assess the clinical use of *RASGRF1* methylation for prediction of gastric cancer. *RASGRF1* methylation was highly discriminative between noncancerous gastric mucosa from patients with gastric cancer and gastric mucosa from healthy individuals (Fig. 3B and Table 2). Earlier studies showed that severe gastric mucosal atrophy induced by *H. pylori* infection is a hallmark of gastric cancer risk, and that

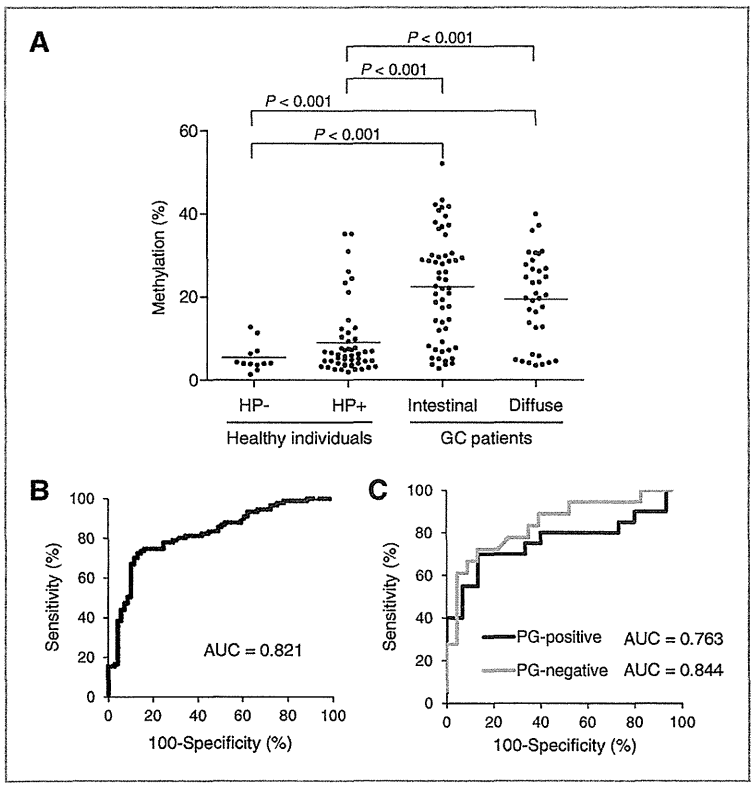

Figure 3. Analysis of *RASGRF1* methylation in gastric mucosae from healthy individuals and patients with gastric cancer. A, summarized results of bisulfite pyrosequencing in normal gastric mucosae from healthy individuals with ($n = 50$) or without *H. pylori* (HP) infection ($n = 12$), and noncancerous gastric mucosae from patients with intestinal type gastric cancer ($n = 55$) or diffuse type gastric cancer ($n = 36$). B, ROC curve analysis of *RASGRF1* methylation. The area under the ROC curve (AUC) for each site conveys its use (in terms of sensitivity and specificity) for distinguishing between noncancerous gastric mucosae from patients with gastric cancer and normal stomach from healthy individuals. C, ROC curve analysis distinguishing between noncancerous gastric mucosae from serum pepsinogen (PG) test-positive (blue) or -negative (red) patients with gastric cancer and normal stomach from healthy individuals.