syndrome, lymphoma, and normal bone marrow specimens. Methylation of 11 genes was frequently detected even in control tissues (Fig. 1C and D). On the other hand, there were 9 genes whose methylation was only detected in a subset of multiple myeloma specimens. The remaining 2 genes showed no methylation, indicating that they were silenced by mechanisms other than DNA methylation.

*Epigenetic inactivation of RASD1 in multiple myeloma cell lines.* Among the 9 genes showing multiple myeloma-specific methylation, we selected RASD1 for further analysis because it was originally detected based on its induction by dexamethasone (26). We initially confirmed the methylation status of RASD1 using bisulfite sequencing and bisulfite pyrosequencing. The results of the bisulfite sequencing were consistent with the methylation levels determined by pyrosequencing (Fig. 2B and C), and there was a significant correlation between the results of pyrosequencing and those of the combined bisulfite restriction analysis ($r$ = 0.905, $P$ = 0.001; rs = 0.854, $P$ = 0.003). Moreover, levels of RASD1 expression determined by real-time quantitative reverse transcription-PCR were inversely correlated with the methylation levels determined by pyrosequencing ($r$ = -0.766, $P$ = 0.010; rs = -0.842, $P$ = 0.002; Fig. 2D). All cell lines with methylated RASD1 exhibited >5-fold increase in RASD1 expression after 5-aza-2′-deoxycytidine treatment, and the induction of RASD1 following 5-aza-2′-deoxycytidine treatment was correlated with the methylation level before treatment ($r$ = 0.645, $P$ = 0.044; rs = 0.733, $P$ = 0.16).

We next carried out real-time PCR with and without dexamethasone treatment to assess the degree to which RASD1 is induced by dexamethasone in multiple myeloma cell lines (Fig. 3A and B). RPMI8226 cells, in which RASD1 is unmethylated, showed the greatest increase in expression (100 nmol/L dexamethasone versus mock). When we then tested whether 5-aza-2′-deoxycytidine would enhance the induction of RASD1 by dexamethasone in multiple myeloma cell lines showing RASD1 methylation, we found that 5-aza-2′-deoxycytidine acted synergistically with dexamethasone to induce RASD1 expression in all of the cell lines tested (Fig. 3B).

*5-Aza-2-deoxycytidine acts synergistically with dexamethasone to suppress dexamethasone-resistant OPM1 cell viability.* Although multiple myeloma cells are generally sensitive to dexamethasone treatment, tolerance appears during the end stage of the disease. Given that RASD1 reportedly suppresses cell growth (27), we hypothesized that dexamethasone acts through activation of RASD1. To test that idea, we first carried out a set of WST-8 assays to determine the dexamethasone sensitivity of each multiple myeloma cell line. Two cell lines (RPMI8226 and MM.1S) with unmethylated RASD1 were clearly sensitive to dexamethasone, which dose-dependently suppressed their viability (Fig. 4A and B). Consistent with their lack of RASD1 methylation, 5-aza-2′-deoxycytidine had no effect on the viability of these two cell lines (Fig. 4B). By contrast, cell lines with methylated RASD1 showed no sensitivity to dexamethasone treatment (Fig. 4A), suggesting that dexamethasone resistance is associated with RASD1 methylation. That finding prompted us to test whether demethylating RASD1 using 5-aza-2′-deoxycytidine would alter the dexamethasone sensitivity of RASD1-methylated cell lines. We found that, in the absence of 5-aza-2′-deoxycytidine, OPM1 cells were insensitive to dexamethasone treatment as reported previously (ref. 8; Fig. 4A). In the presence of 5-aza-2′-deoxycytidine, however, dexamethasone suppressed OPM1 cell viability in a time- and dose-dependent manner (Fig. 4C). Thereafter, we compared the sensitivities to dexamethasone and 5-aza-2′-deoxycytidine treatment of two drug-resistant cell lines derived from the dexamethasone-sensitive cell lines shown in Fig. 4B, taking into consideration the differences in their methylation status. RPMI8226/Dox40 cells are

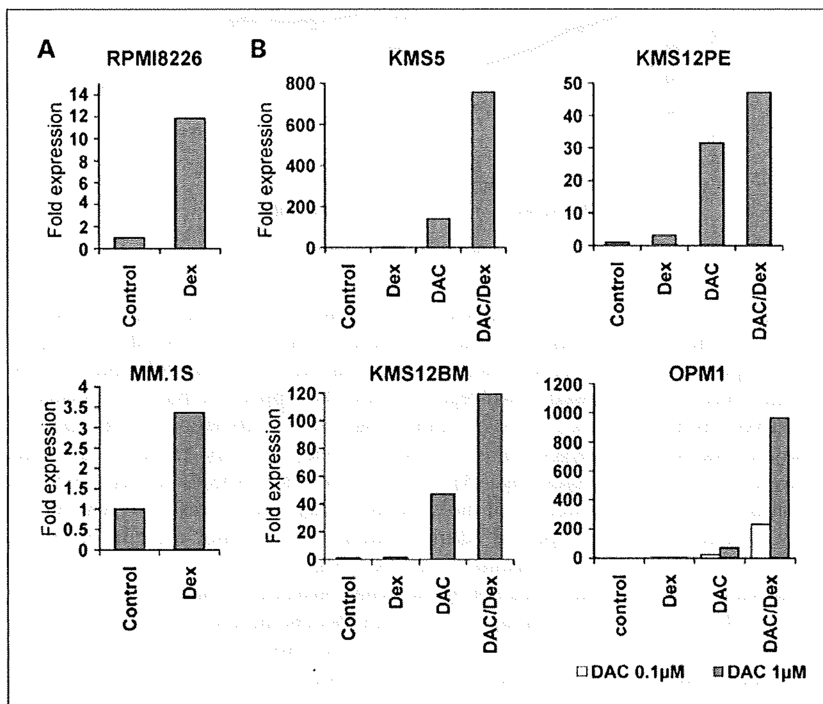


Fig. 3. RASD1 expression was synergistically induced by 5-aza-2′-deoxycytidine + dexamethasone in cell lines with methylated RASD1 alleles. *A,* fold expression of RASD1 (100 nmol/L dexamethasone/mock) with or without dexamethasone treatment in cell lines with unmethylated RASD1. *B,* fold RASD1 expression with dexamethasone, 5-aza-2′-deoxycytidine, or 5-aza-2′-deoxycytidine + dexamethasone versus control in multiple myeloma cell lines exhibiting RASD1 methylation.

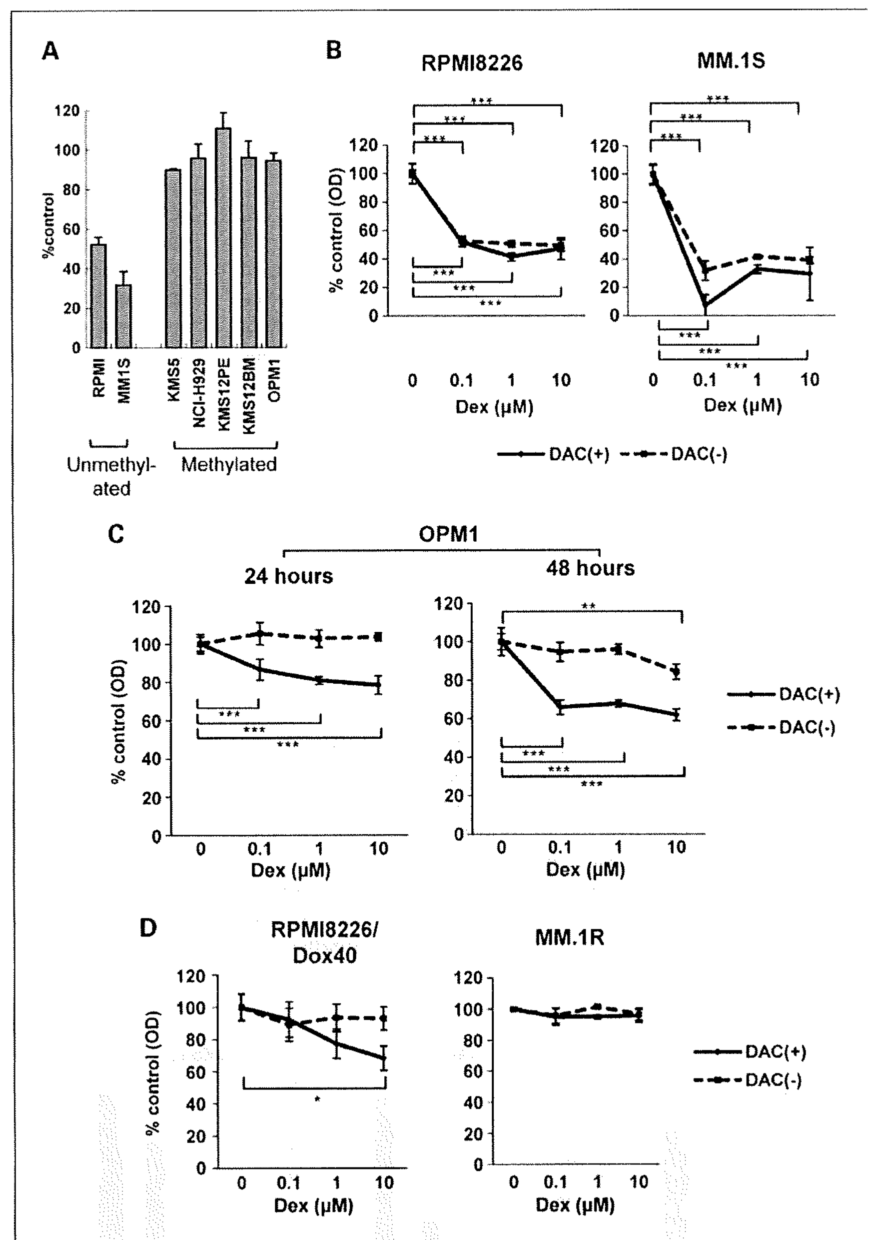


**Fig. 4.** Treatment with 5-aza-2′-deoxycytidine + dexamethasone synergistically suppressed dexamethasone-resistant OPM1 cell viability. *A,* cell lines with methylated RASD1 showed dexamethasone resistance in WST-8 assays. *B,* two cell lines (RPMI8226 and MM.1S) with unmethylated RASD1 were suppressed by dexamethasone (0, 0.1, 1, or 10 μmol/L). There was no difference between cell viabilities obtained with and without 5-aza-2′-deoxycytidine. *C,* treatment with 5-aza-2′-deoxycytidine + dexamethasone treatment synergistically suppressed OPM1 cell viability in a time- and dose-dependent manner. *D,* treatment with 5-aza-2′-deoxycytidine + dexamethasone synergistically suppressed the viability of RPMI8226/Dox40 cells but not MM.1R cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (one-way ANOVA with post hoc Dunnett's test).

doxorubicin-resistant cells derived from the RPMI8226 line; RASD1 is highly methylated (95%; data not shown) in these cells, and like OPM1 cells, their viability was suppressed by treatment with 5-aza-2′-deoxycytidine + dexamethasone (Fig. 4D). By contrast, MM.1R cells, which are derived from MM.1S cells, are known to be dexamethasone-resistant due to glucocorticoid receptor truncation, not RASD1 methylation (30, 31). Consistent with the absence of RASD1 methylation (<5%; data not shown), 5-aza-2′-deoxycytidine had no ability to enhance dexamethasone cytotoxicity toward MM.1R cells (Fig. 4D).

*Increased RASD1 methylation after repeated treatments in multiple myeloma patients.* To assess the levels of RASD1 methylation in primary multiple myeloma cells, we performed pyrosequencing using CD138$^+$ cells from 87 multiple myeloma patients and 12 control subjects without tumors (Fig. 5A and B). We selected 10% as the cutoff for methylation based on our findings that it represents the 75th percentile among the control samples and that cell lines with methylation of >10% showed down-regulated RASD1 expression that was restored by 5-aza-2′-deoxycytidine (Fig. 2B). Methylation of RASD1 was observed in 8 of the 87 (8%) primary multiple myeloma samples (Fig. 5B). Moreover, levels of RASD1 methylation were elevated in all multiple myeloma cases (5 cases) in which there was repeated administration of antitumor therapy, including dexamethasone ($P < 0.001$; Fig. 5C).

*Identification of the genes involved in the synergistic effect of 5-aza-2-deoxycytidine with dexamethasone.* To identify genes responsible for the synergistic effect of 5-aza-2′-deoxycytidine with dexamethasone, we performed cDNA microarray analyses using cDNA prepared from dexamethasone-resistant OPM1

cells treated with or without dexamethasone, 5-aza-2′-deoxycytidine, or 5-aza-2′-deoxycytidine + dexamethasone. We then selected genes whose expression was up-regulated or down-regulated >3-fold by 5-aza-2′-deoxycytidine + dexamethasone for analysis of gene tree clustering (Fig. 6A; Supplementary Table S4). One cluster exhibited an expression pattern similar to that of RASD1 shown in Fig. 3B. This cluster included RASD1 as well as other known cancer-related genes (Supplementary Table S4). To validate the results of the microarray analysis, we then carried out quantitative real-time PCR for four genes chosen from this cluster (Fig. 6B), and the results were consistent with those obtained with the microarray. We also examined the expression of SOCS3, which plays a role in the regulation of the interleukin-6 signaling pathway. Like that of RASD1, expression of SOCS3 was synergistically up-regulated in cells treated with 5-aza-2′-deoxycytidine + dexamethasone (Fig. 6B).

Finally, we performed bisulfate sequencing analysis to examine the role of DNA methylation in silencing genes up-regulated by 5-aza-2′-deoxycytidine + dexamethasone (Supplementary Fig. S5). Significant methylation of BNIP3 was detected, although methylation of SOCS3 gene was not, and ROS1 does not contain a CpG island in its 5′ region. Thus 5-aza-2′-deoxycytidine + dexamethasone appears to suppress cell growth in both DNA methylation-dependent and DNA methylation-independent manners.

## Discussion

Although genomic screening of genes silenced by DNA methylation has been reported previously (14, 16), the epigenetic alterations involved in tumorigenesis of multiple myeloma are still not fully understood. In the present study, genomic screening revealed that 128 genes were up-regulated >10-fold by 5-aza-2′-deoxycytidine in RPMI8226 cells, and 83 were similarly up-regulated in KMS12-PE cells, which is comparable with earlier findings (14). In addition, we found that 5-aza-2′-deoxycytidine up-regulated 424 genes >10-fold in OPM1 cells. This suggests that different numbers of genes are targeted for methylation in different multiple myeloma cells. This implies that the utility of the expression-based approach to finding target genes silenced by DNA methylation is limited because (*a*) 5-aza-2′-deoxycytidine induces expression of genes silenced via multiple mechanisms (e.g., DNA damage and growth inhibition) and (*b*) the sensitivity of microarrays is limited. We therefore focused on genes that have CpG islands in their 5′ regions using real-time PCR to confirm restoration of their expression by 5-aza-2′-deoxycytidine and bisulfite PCR to assess their methylation status. We found specific methylation of 9 genes in multiple myeloma cells and tissue specimens but not in normal bone marrow cells, suggesting that these 9 genes are involved in multiple myeloma tumorigenesis.

RASD1 was originally identified as a dexamethasone-inducible gene (26), and its product was shown to be a receptor-independent activator of G-protein signaling (32, 33). RASD1 protein belongs to the Ras family (e.g., RIG, ARH1/NOEY2, and RRP22), which was recently shown to suppress cell growth (34–36). Located on chromosome 17p11.2, loss of RASD1 heterozygosity is frequently detected in human tumors, and Furuta et al. reported epigenetic inactivation of RASD1 in a melanoma cell line (37), which suggests that inactivation of RASD1 provides a growth advantage to tumor cells. In the

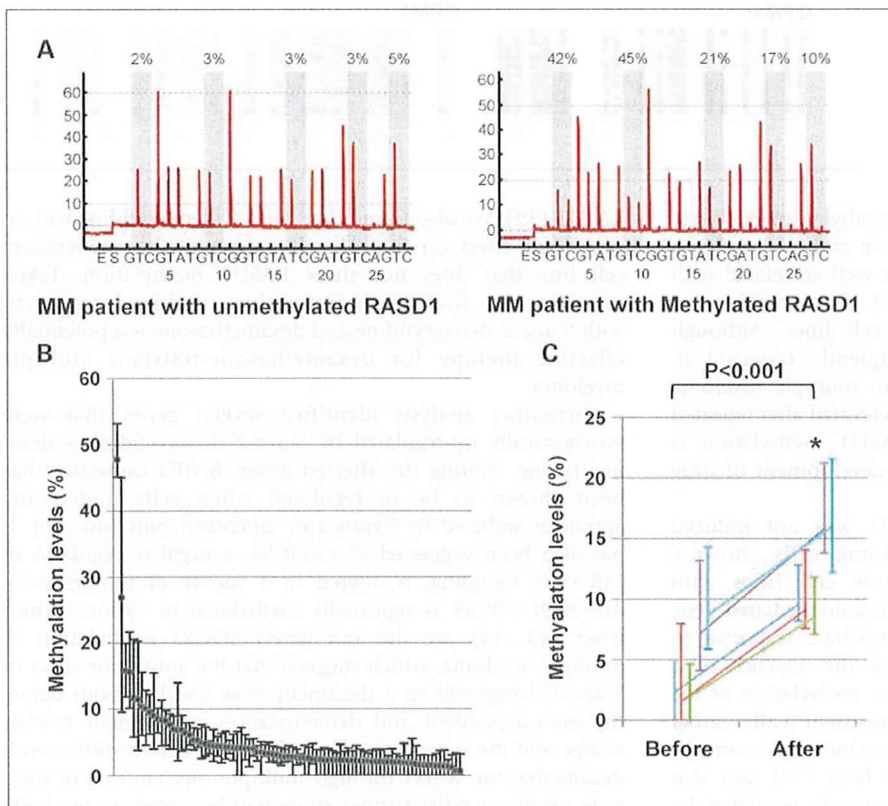


Fig. 5. Although levels of RASD1 methylation in primary multiple myeloma samples were lower than in multiple myeloma cell lines, RASD1 methylation was increased after repeated treatments in multiple myeloma patients. *A*, examples of pyrograms in CD138$^+$ cells collected from primary multiple myeloma patients (methylated and unmethylated RASD1). *B*, RASD1 methylation levels in CD138$^+$ cells collected from 87 primary multiple myeloma patients. Mean RASD1 methylation levels (*black box*) with maximum and minimum levels (*vertical lines*) at five CpG sites in the RASD1 promoter region. *C*, mean RASD1 methylation levels with maximum and minimum levels at five CpG sites in the RASD1 promoter region before or after repeated antitumor treatments. Five patients received dexamethasone therapy. Of those, three received bortezomib with dexamethasone, one received vincristine, Adriamycin with dexamethasone (*VAD*), and one received bortezomib with dexamethasone and high-dose melphalan followed by peripheral blood stem cell transplantation. RASD1 methylation was elevated after repeated treatment in multiple myeloma patients. $P < 0.001$ (paired $t$ test).

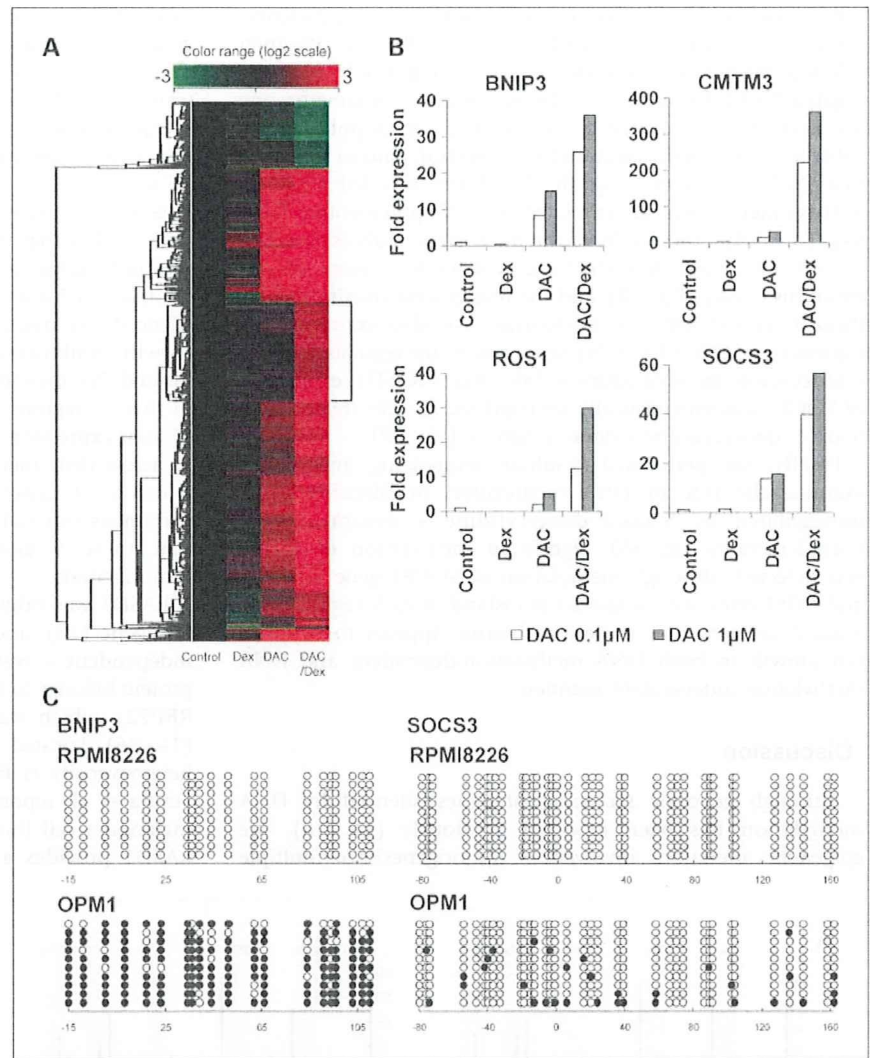


**Fig. 6.** cDNA microarray analysis suggests that there is a group of genes that could be involved in the synergistic effect seen with 5-aza-2′-deoxycytidine + dexamethasone. *A,* cDNA microarray analysis with or without dexamethasone, 5-aza-2′-deoxycytidine, or 5-aza-2′-deoxycytidine + dexamethasone. A heat map created by gene tree clustering of genes showing >3-fold up-regulation or down-regulation in their expression with 5-aza-2′-deoxycytidine + dexamethasone. These genes were selected after per-chip normalization (normalized to 50th percentile) and per-gene normalization (normalized to genes in a control sample). *B,* results of real-time quantitative PCR with or without dexamethasone, 5-aza-2′-deoxycytidine, or 5-aza-2′-deoxycytidine + dexamethasone. BNIP3, CMTM3, and ROS1 were chosen from the cluster in *A*. SOCS3, a negative regulator of IL-6 signaling pathway, was also examined. *C,* bisulfite sequencing analysis of BNIP3 and SOCS3 in multiple myeloma cell lines.

present study, we showed that RASD1 methylation is closely associated with dexamethasone resistance in multiple myeloma cells. Levels of RASD1 methylation were well correlated with the silencing of RASD1 and impaired the sensitivity to dexamethasone in multiple myeloma cell lines. Although RASD1 hypermethylation was less frequently observed in primary multiple myeloma samples than multiple myeloma cell lines, methylation levels were always elevated after repeated antitumor therapy, suggesting that RASD1 methylation is pivotal for disease progression and the development of drug resistance in multiple myeloma.

We found that expression of RASD1 was not induced by dexamethasone in multiple myeloma cells showing RASD1 methylation, but treating these cell lines with 5-aza-2′-deoxycytidine restored dexamethasone-mediated gene expression. This indicates that the impaired response to dexamethasone need not be caused by the absence of a transcription factor but can be caused by methylation of the RASD1 promoter. Our findings are consistent with earlier reports that inhibition of DNA methylation induces dexamethasone-sensitive clones in lymphoid cell lines (38) and that B-cell proliferation and activity are negatively regulated by RASD1 (39). We also found that 5-aza-2′-deoxycytidine had no significant effect on MM.R1 cells, a dexamethasone-resistant cell line that does not show RASD1 methylation. Taken together, our findings indicate that combined treatment with 5-aza-2′-deoxycytidine and dexamethasone is a potentially effective therapy for dexamethasone-resistant multiple myeloma.

Microarray analysis identified several genes that were synergistically up-regulated by 5-aza-2′-deoxycytidine + dexamethasone. Among the affected genes, BNIP3 expression has been shown to be up-regulated when cells undergoing apoptosis induced by hypoxia or glucocorticoids (40, 41). It has also been suggested that SOCS3, a negative regulator of JAK-STAT signaling, is altered in a variety of tumors (42). Although SOCS3 is reportedly methylated in various tumor types (43, 44), we did not detect SOCS3 methylation in multiple myeloma, which suggests that the antitumor effect of 5-aza-2′-deoxycytidine + dexamethasone involves both demethylation-dependent and demethylation-independent mechanisms, and the synergistic effect of 5-aza-2′-deoxycytidine with dexamethasone works through multiple mechanisms in multiple myeloma cells. Further study will be necessary to clarify

the molecular mechanisms underlying the synergistic effect of 5-aza-2′-deoxycytidine with dexamethasone.

In conclusion, we have shown that epigenetic inactivation of RASD1 is closely correlated with resistance to dexamethasone in multiple myeloma cells. Restoration of RASD1 expression in multiple myeloma cells using 5-aza-2′-deoxycytidine also restored sensitivity to dexamethasone. Although further study is needed to determine how important RASD1 hypermethylation is in the clinical course of multiple myeloma, our results are indicative of the potential utility of demethylation therapy in cases of advanced multiple myeloma.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

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## References

1. Child JA, Morgan GJ, Davies FE, et al. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. N Engl J Med 2003;348:1875–83.
2. Gehring U, Mohit B, Tomkins GM. Glucocorticoid action on hybrid clones derived from cultured myeloma and lymphoma cell lines. Proc Natl Acad Sci U S A 1972;69:3124–7.
3. Smith L, Alexanian R. Treatment strategies for plasma cell myeloma. CA Cancer J Clin 1985;35:214–20.
4. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res 2001;61:3071–6.
5. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 2008;14:1649–57.
6. Palumbo A, Gay F, Bringhen S, et al. Bortezomib, doxorubicin and dexamethasone in advanced multiple myeloma. Ann Oncol 2008;19:1160–5.
7. Pineda-Roman M, Zangari M, van Rhee F, et al. VTD combination therapy with bortezomib-thalidomide-dexamethasone is highly effective in advanced and refractory multiple myeloma. Leukemia 2008;22:1419–27.
8. Neri P, Yasui H, Hideshima T, et al. *In vivo* and *in vitro* cytotoxicity of *R*-etodolac with dexamethasone in glucocorticoid-resistant multiple myeloma cells. Br J Haematol 2006;134:37–44.
9. Yasui H, Hideshima T, Hamasaki M, et al. SDX-101, the *R*-enantiomer of etodolac, induces cytotoxicity, overcomes drug resistance, and enhances the activity of dexamethasone in multiple myeloma. Blood 2005;106:706–12.
10. Yasui H, Hideshima T, Raje N, et al. FTY720 induces apoptosis in multiple myeloma cells and overcomes drug resistance. Cancer Res 2005;65:7478–84.
11. Gonzalez D, van der Burg M, Garcia-Sanz R, et al. Immunoglobulin gene rearrangements and the pathogenesis of multiple myeloma. Blood 2007;110:3112–21.
12. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood 2004;104:607–18.
13. De Bruyne E, Bos TJ, Asosingh K, et al. Epigenetic silencing of the tetraspanin CD9 during disease progression in multiple myeloma cells and correlation with survival. Clin Cancer Res 2008;14:2918–26.
14. Heller G, Schmidt WM, Ziegler B, et al. Genome-wide transcriptional response to 5-aza-2′-deoxycytidine and trichostatin A in multiple myeloma cells. Cancer Res 2008;68:44–54.
15. Kroeger H, Jelinek J, Estecio MR, et al. Aberrant CpG island methylation in acute myeloid leukemia is accentuated at relapse. Blood 2008;112:1366–73.
16. Pompeia C, Hodge DR, Plass C, et al. Microarray analysis of epigenetic silencing of gene expression in the KAS-6/1 multiple myeloma cell line. Cancer Res 2004;64:3465–73.
17. Takahashi T, Shivapurkar N, Reddy J, et al. DNA methylation profiles of lymphoid and hematopoietic malignancies. Clin Cancer Res 2004;10:2928–35.
18. Issa JP. DNA methylation as a therapeutic target in cancer. Clin Cancer Res 2007;13:1634–7.
19. Issa JP, Garcia-Manero G, Giles FJ, et al. Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 2004;103:1635–40.
20. Oki Y, Jelinek J, Shen L, Kantarjian HM, Issa JP. Induction of hypomethylation and molecular response after decitabine therapy in patients with chronic myelomonocytic leukemia. Blood 2008;111:2382–4.
21. Eramo A, Pallini R, Lotti F, et al. Inhibition of DNA methylation sensitizes glioblastoma for tumor necrosis factor-related apoptosis-inducing ligand-mediated destruction. Cancer Res 2005;65:11469–77.
22. Fojo T, Bates S. Strategies for reversing drug resistance. Oncogene 2003;22:7512–23.
23. Murgo AJ. Innovative approaches to the clinical development of DNA methylation inhibitors as epigenetic remodeling drugs. Semin Oncol 2005;32:458–64.
24. Satoh A, Toyota M, Itoh F, et al. Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. Cancer Res 2003;63:8606–13.
25. Shen L, Kondo Y, Ahmed S, et al. Drug sensitivity prediction by CpG island methylation profile in the NCI-60 cancer cell line panel. Cancer Res 2007;67:11335–43.
26. Kemppainen RJ, Behrend EN. Dexamethasone rapidly induces a novel ras superfamily member-related gene in AtT-20 cells. J Biol Chem 1998;273:3129–31.
27. Vaidyanathan G, Cismowski MJ, Wang G, Vincent TS, Brown KD, Lanier SM. The Ras-related protein AGS1/RASD1 suppresses cell growth. Oncogene 2004;23:5858–63.
28. Estecio MR, Gharibyan V, Shen L, et al. LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. PLoS ONE 2007;2:e399.
29. Nojima M, Suzuki H, Toyota M, et al. Frequent epigenetic inactivation of SFRP genes and constitutive activation of Wnt signaling in gastric cancer. Oncogene 2007;26:4699–713.
30. Moalli PA, Pillay S, Weiner D, Leikin R, Rosen ST. A mechanism of resistance to glucocorticoids in multiple myeloma: transient expression of a truncated glucocorticoid receptor mRNA. Blood 1992;79:213–22.
31. Sharma S, Lichtenstein A. Dexamethasone-induced apoptotic mechanisms in myeloma cells investigated by analysis of mutant glucocorticoid receptors. Blood 2008;112:1338–45.
32. Cismowski MJ, Takesono A, Ma C, et al. Genetic screens in yeast to identify mammalian nonreceptor modulators of G-protein signaling. Nat Biotechnol 1999;17:878–83.
33. Takesono A, Cismowski MJ, Ribas C, et al. Receptor-independent activators of heterotrimeric G-protein signaling pathways. J Biol Chem 1999;274:33202–5.
34. Elam C, Hesson L, Vos MD, et al. RRP22 is a farnesylated, nucleolar, Ras-related protein with tumor suppressor potential. Cancer Res 2005;65:3117–25.
35. Ellis CA, Vos MD, Howell H, Vallecorsa T, Fults DW, Clark GJ. Rig is a novel Ras-related protein and potential neural tumor suppressor. Proc Natl Acad Sci U S A 2002;99:9876–81.
36. Yu Y, Xu F, Peng H, et al. NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. Proc Natl Acad Sci U S A 1999;96:214–9.
37. Furuta J, Nobeyama Y, Umebayashi Y, Otsuka F, Kikuchi K, Ushijima T. Silencing of peroxiredoxin 2 and aberrant methylation of 33 CpG islands in putative promoter regions in human malignant melanomas. Cancer Res 2006;66:6080–6.
38. Gasson JC, Bourgeois S. A new determinant of glucocorticoid sensitivity in lymphoid cell lines. J Cell Biol 1983;96:409–15.
39. Lindsey JW. Dexamethasone-induced Ras-related protein 1 is a potential regulatory protein in B lymphocytes. Int Immunol 2007;19:583–90.
40. Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A 2000;97:9082–7.
41. Sandau US, Handa RJ. Glucocorticoids exacerbate hypoxia-induced expression of the pro-apoptotic gene Bnip3 in the developing cortex. Neuroscience 2007;144:482–94.
42. Kubo M, Hanada T, Yoshimura A. Suppressors of cytokine signaling and immunity. Nat Immunol 2003;4:1169–76.
43. He B, You L, Uematsu K, et al. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci U S A 2003;100:14133–8.
44. Niwa Y, Kanda H, Shikauchi Y, et al. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. Oncogene 2005;24:6406–17.

*Review Article*

# Cancer epigenomics: Implications of DNA methylation in personalized cancer therapy

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**Genetic alterations in cancer can provide information for predicting a tumor's sensitivity to chemotherapeutic drugs. But although such information is certainly useful, the relatively low frequency of mutations seen in many cancers limits the utility of pharmacogenomics in large numbers of cancer patients, necessitating consideration of other approaches. Epigenetic changes such as DNA methylation are a hallmark of human cancers. Methylation of genes involved in DNA repair and maintaining genome integrity (e.g. *MGMT, hMLH1, WRN,* and *FANCF*), and cell-cycle checkpoint genes (e.g. *CHFR* and *14-3-3 σ, CDK10,* and *p73*), all reportedly influence the sensitivity to chemotherapeutic drugs, suggesting that DNA methylation could serve as a molecular marker for predicting the responsiveness of tumors to chemotherapy. However, the comprehensive study of pharmacoepigenomics awaits the advent of genome-wide analysis of DNA methylation using microarrays and next-generation sequencers. (*Cancer Sci* 2009; 100: 787–791)**

Cancer arises through the accumulation of multiple genetic changes, including point mutations, gene amplifications and gene deletions, which ultimately lead to activation of oncogenes and inactivation of tumor-suppressor genes.[1] Moreover, it was recently proposed that cancer cells are 'addicted' to oncogenes for maintenance of the malignant phenotype.[2] The most convincing evidence for the concept of oncogene addiction comes from the increasing number of studies showing the therapeutic efficacy of antibodies and drugs that selectively target specific oncogenes in human cancers. For example, mutation of *EGFR* indicates sensitivity to gefitinib,[3–5] the presence of *BCR-ABL* translocation or mutation of c-kit indicates sensitivity to imatinib,[6] and amplification or overexpression of human EGFR-related 2 (*Her-2*)/*ErbB2* indicates sensitivity to herceptin.[7] Thus genetic alterations in cancer can provide important information that enables one to predict the sensitivity of a given tumor to particular chemotherapeutic drugs.

Information about gene expression also can be used to predict the response to chemotherapy. Profiles of gene expression in cancer cell lines revealed an association between the expression of certain genes and the cells' sensitivity to chemotherapeutic drugs.[8,9] What's more, gene expression signatures have been used clinically to predict the likely responsiveness of tumors to chemotherapy.[10] But although information about genetic changes certainly contributes to our ability to predict sensitivity to chemotherapeutic drugs, the relatively low frequency of mutations seen in many cancers limits the utility of pharmacogenomics in large numbers of cancer patients, necessitating consideration of other approaches. In this review, we focus on the implications of epigenetic alterations such as DNA methylation in predicting the efficacy of chemotherapeutic drugs in the treatment of cancer.

## Role of DNA methylation in carcinogenesis

DNA methylation of the 5′-CpG islands of genes plays an important role in gene regulation. Under normal physiological conditions, DNA methylation is involved in regulating genome imprinting, X-chromosome inactivation, and inactivation of repetitive sequences. Three DNA methyltransferases (DNMT1, DNMT3A, DNMT3B) catalyze methylation of the promoter regions of a variety of genes, including genes involved in cell-cycle checkpoints, apoptosis, DNA repair, cell adhesion, and signal transduction.[11–13] Simultaneous methylation of multiple genes occurs in colorectal cancers that show the CIMP,[14] and the majority of sporadic colorectal cancers that show microsatellite instability are associated with CIMP, which leads to inactivation of the mismatch repair gene *hMLH1* and thus disruption of mismatch repair.[15] DNA methylation also plays a role in altering signaling pathways in cancer. For example, epigenetic inactivation of *SFRP1*, *SFRP2*, *SFRP5*, *DKK1*, *DKK2*, and *DKK3*, six negative regulators of WNT signaling, contributes to the full activation of T cell Factor (TCF) β-catenin activity in colorectal cancers (Fig. 1a,b),[16,17] whereas epigenetic inactivation of *RASSF1* and *RASSF2*, negative regulators of the Ras signaling pathway, contributes to full activation of oncogenic Ras signaling (Fig. 2).[18,19] Although the molecular mechanisms underlying DNA methylation remain unclear, recent studies suggest that inflammation and pathogens are likely involved.[20,21]

## Epigenetic inactivation of DNA repair and altered sensitivity to chemotherapeutic drugs

Genomic instability is an important phenotype that allows cancer cells to generate oncogenic translocations, inactivate tumor-suppressor genes, and amplify oncogenes and drug-resistance genes. Genomic instability is caused by impairment or inactivation of DNA repair systems, which could represent a molecular target of cancer therapy. Evidence suggests, for example, that epigenetic inactivation of DNA repair underlies tumor responsiveness to DNA-damaging agents. The first reported epigenetic alteration associated with sensitivity to a chemotherapeutic drug was the association between methylation of the *MGMT* gene and sensitivity to alkylating agents.[22] MGMT is a DNA repair

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Abbreviations: 5-aza-dC, 5-aza-2′-deoxycitidine; 5-FU, 5-fluorouracil; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BCR-ABL, breakpoint cluster region-ABL; CDK10, cyclin-dependent kinase 10; CHFR, checkpoint with ring finger; CIMP, CpG island methylator phenotype; DNMT, DNA methyltransferase; EGFR, epidermal growth factor receptor; FA, Fanconi anemia; FANCF, Fanconi anemia protein F; hMLH1, human mutL homolog 1; MAPK, mitogenactivated protein kinase; MGMT, $O^6$-methylguanine-DNA-methyltransferase; RASSF, Ras association domain family; SFRP, secreted frizzled-related protein; shRNA, short hairpin RNA; siRNA, short interfering RNA; WRN, Werner syndrome protein.

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enzyme that removes mutagenic adducts from $O^6$-guanine in DNA,[23] and its epigenetic silencing has been reported in a wide variety of tumors.[24] This silencing of *MGMT* is associated with G : C to A : T transition mutations in K-ras and p53, a mutator phenotype distinct from mismatch repair deficiency.[25,26] Alkylating agents are one of the most widely used classes of chemotherapeutic drugs and frequently act by modifying the $O^6$ position of guanine. Consequently, their toxicity, and thus their efficacy, is diminished in tumors expressing MGMT.[27] For example, Esteller *et al.* reported that *MGMT* gene methylation correlates with response of gliomas to BCNU (Fig. 3).[22] Moreover, several clinical trials have shown *MGMT* gene methylation to be an independent predictor of outcome in glioblastoma patients treated with methylating agents.[28,29]

Approximately 15% of colorectal cancers show microsatellite instability due to methylation of the mismatch repair gene *hMLH1*.[14] Clinically, colorectal cancers with *hMLH1* methylation are less aggressive, but they do not respond to 5-FU.[30] Thymidylate synthase catalyzes the conversion of dUMP to dTMP, which is necessary for DNA synthesis, and inhibition of this enzyme is the major mechanism underlying the anticancer effects of 5-FU. Ricciardiello *et al.* reported that colorectal cancers with *hMLH1* methylation express high levels thymidylate synthase.[31] Moreover, colorectal cancer cell lines displaying microsatellite instability are resistant to 5-FU due to methylation of *hMLH1*, but they become susceptible to treatment upon exposure to 5-aza-dC.[32] Thus methylation of *hMLH1* appears to be a predictive molecular marker of the sensitivity of colorectal cancers to 5-FU.

RecQ-like helicases also reportedly play a role in the maintenance of genetic stability, and disruption of their activity results in chromosome breakage syndromes such as Bloom syndrome, Rothmund–Thomson syndrome, and Werner syndrome, the last of which is an inherited disorder characterized by the premature onset of aging and susceptibility to various types of cancer. Recently, Agrelo *et al.* reported that the *WRN* gene is frequently silenced by DNA methylation in colorectal cancers[33] and that colorectal cancer cell lines showing *WRN* methylation are sensitive to the topoisomerase inhibitor camptothecin and to the interstrand crosslinker mitomycin C. Clinically, moreover, colorectal cancers exhibiting *WRN* methylation respond well to the topoisomerase inhibitor irinotecan. Hypermethylation of *WRN* in colorectal tumors could thus be a useful predictor of a robust clinical response to a topoisomerase inhibitor.

Fanconi anemia is an autosomal recessive chromosomal instability syndrome that causes FA patients to be prone to various types of malignancies. Taniguchi *et al.* reported that epigenetic inactivation of one of the FA complementation group genes, *FANCF*, is associated with resistance to cisplatin.[34] Defects in the FA–Breast Cancer (BRCA) pathway are associated with genomic instability and increased sensitivity to DNA-damaging agents such as mitomycin C and cisplatin, and there is a significant correlation between *FANCF* methylation and sensitivity to cisplatin in ovarian cancer cell lines, so that restoration of *FNCAF* expression using 5-aza-dC induces resistance to cisplatin. Methylation of *FANCF* has been found in 20% of primary ovarian cancers not previously exposed to cisplatin,[34] but the correlation between chemosensitivity and *FANCF* methylation in primary tumors remains to be determined. Methylation of *FANCF* was also found in 30% of cervical cancers, 15% of head and neck squamous cell cancers, and 14% of non-small cell lung cancers.[35,36] Further study will be necessary to determine whether methylation of *FANCF* is a predictive marker of sensitivity to DNA-damaging agents.

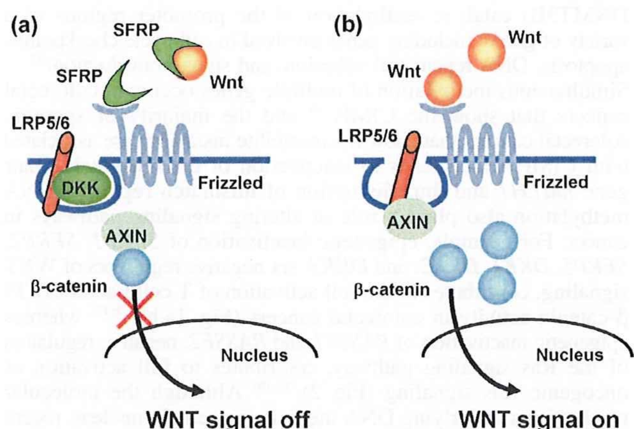


**Fig. 1.** Epigenetic inactivation of negative regulators of WNT signaling. (a) In normal cells, SFRP and DKK are associated with key WNT signaling molecules such as WNT ligands and LRP5/6, which prevent translocation of β-catenin to the nucleus. (b) In cancer cells, epigenetic inactivation of SFRP and DKK enables β-catenin to translocate to the nucleus, which leads to activation of WNT signaling. SFRP, secreted frizzled-related protein; DKK, Dickkopf; LRP, lipoprotein receptor-related protein.

## Cell-cycle checkpoint defects and sensitivity to chemotherapeutic drugs

Impairment of cell-cycle checkpoints is associated with sensitivity to chemotherapeutic agents. For example, overexpression of mitotic arrest difficient 2 (MAD2) sensitizes cancer cells to both cisplatin and vincristine,[37,38] whereas overexpression of Aurora A induces chemoresistance.[39] In addition, we recently found that

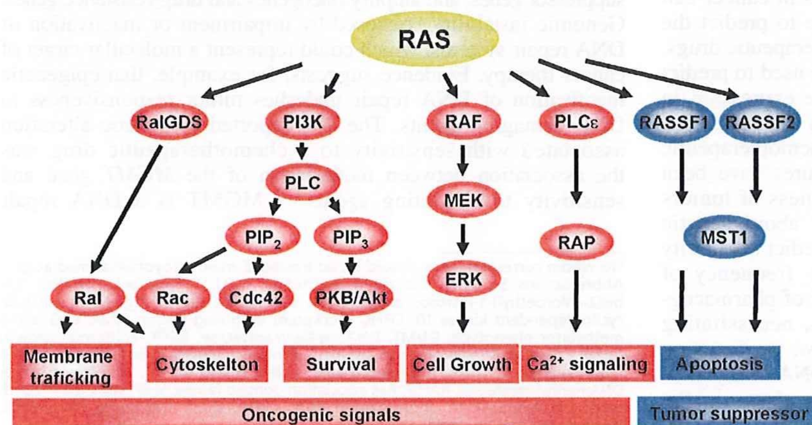


**Fig. 2.** Positive and negative regulators of Ras signaling. The oncogenic and anti-oncogenic functions of Ras are mediated by positive and negative effectors. Among the negative effectors of Ras, epigenetic inactivation of RASSF1 and RASSF2 is frequently observed in human tumors. Akt, v-akt murine thymoma viral oncogene homolog; Cdc, cell-division cycle; Erk, extracellular signal-regulated kinase; MEK, Mitogen-activated protein kinase; MST, mammalian STE20-like protein kinase 1; PI3K, Phosphoinositide 3-kinase; PIP, phosphatidylinositol phosphate; PKB, protein kinase B; RAP, ras-related protein; RASSF, Ras association domain family.



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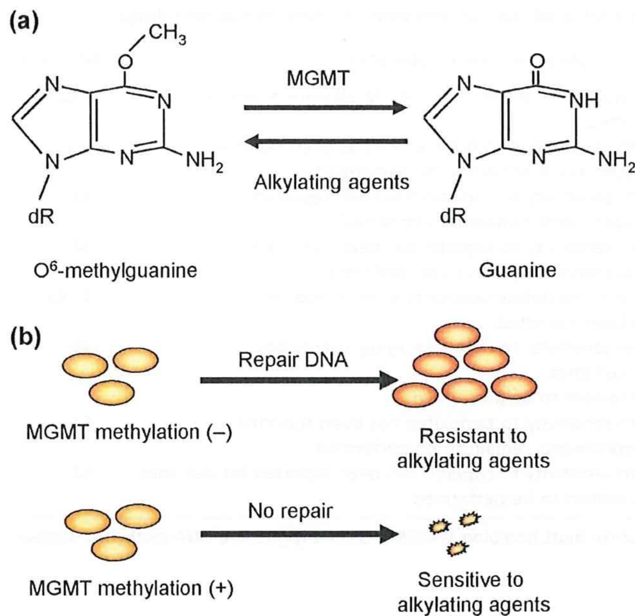


**Fig. 3.** Epigenetic inactivation of $O^6$-methylguanine-DNA-methyltransferase (MGMT) and sensitivity to alkylating agents. (a) MGMT repairs $O^6$-methylguanine. (b) Cancers with *MGMT* methylation are sensitive to alkylating agents due to the absence of $O^6$-methylguanine repair activity.

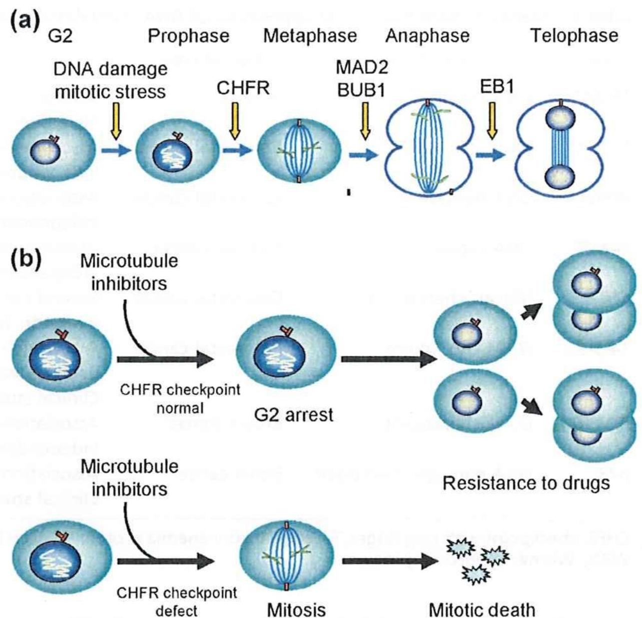


**Fig. 4.** Epigenetic inactivation of a mitotic checkpoint gene, *CHFR*, and sensitivity to microtubule inhibitors. (a) Genes involved in the mitotic checkpoint. (b) CHFR and sensitivity to microtubule inhibitors. Cancer cells that show an intact CHFR checkpoint arrest at $G_2$–M phase after treatment with microtubule inhibitors, which allows the cells to grow. These tumors are resistant to the drugs (top). By contrast, cancer cells that show methylation of *CHFR* do not arrest after treatment with microtubule inhibitors. These tumors are sensitive to the drugs (bottom). BUB, budding uninhibited by benzimidazoles; CHFR, checkpoint with ring finger; EB, end-binding protein; MAD, mitotic arrest deficient.

two microtubule inhibitors, paclitaxel and docetaxel, induce apoptosis among gastric cancer cells showing *CHFR* methylation and that adenoviral introduction of *CHFR* into methylated cancer cell lines restores the checkpoint and reduces the incidence of apoptosis (Fig. 4).[40] This correlation between *CHFR* methylation and sensitivity to microtubule inhibitors appears to be specific, as there was no correlation between *CHFR* methylation and sensitivity to other chemotherapeutic agents (e.g. VP16) or to UV. This suggests that *CHFR* methylation could serve as a clinically useful predictive marker of the sensitivity of tumors to microtubule inhibitors. Consistent with that idea, Koga *et al.* found that six of seven (86%) patients with methylated *CHFR* tumors showed some regression or no progression of their disease when treated with a microtubule inhibitor, whereas four of five (80%) patients with an unmethylated *CHFR* tumor showed progressive deterioration.[41] A correlation between *CHFR* methylation and sensitivity to microtubule inhibitors also was noted in oral squamous cell carcinoma.[42]

The fact that *CHFR* is frequently inactivated by genetic or epigenetic alteration in human cancers suggests that this cancer-specific checkpoint defect also could be a useful therapeutic target.[40,43] Bearing that in mind, we recently established a system to knock down *CHFR* expression using shRNA.[42] We found that *CHFR* expression was significantly suppressed in cancer cells transfected with shRNA, and the resultant impairment of the prophase checkpoint led to an increased mitotic index in cells treated with microtubule inhibitors, which in turn led to an increased incidence of apoptosis. This effect was specific to microtubule inhibitors, as no effect was seen when a DNA-damaging agent (cisplatin or VP16) was used. In addition, an earlier finding that E3 ubiquitin ligases can be targeted using small molecules[44] suggests drugs that inhibit CHFR's ubiquitin ligase activity also could be used to enhance the sensitivity of cancer cells to microtubule inhibitors.

Disruption of the $G_2$–M checkpoint also appears to contribute to the sensitivity of chemotherapeutic drugs. Among the genes involved in the $G_2$–M checkpoint, *14-3-3 σ*, a transcriptional target of p53,[45] is frequently silenced by DNA methylation in breast and gastric cancers,[46,47] and it has been suggested that 14-3-3 σ is a critical regulator of $G_2$–M that also has tumor-suppressor activity. Knocking out *14-3-3 σ* in cancer cells leads to mitotic catastrophe and cell death following DNA damage resulting form the absence of $G_2$–M arrest.[48,49] Consistent with those data, the $G_2$–M checkpoint is impaired in gastric cancer cell lines that show methylation of *14-3-3 σ*,[47] and restoration of *14-3-3 σ* expression using 5-aza-dC restores $G_2$–M arrest induced by DNA damage. In addition, functional proteomic analysis revealed 14-3-3 σ to be a key molecule that contributes to resistance to mitoxantrone and adriamycin in breast cancer cells.[50]

Using high-throughput siRNA screening, Iorns *et al.* identified CDK10 as an important determinant of resistance to endocrine therapy in breast cancer, and thus a major factor limiting successful treatment of the disease.[51] They also found that knocking down CDK10 increases V-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2)-driven transcription of C-Raf, resulting in activation of the MAPK pathway and loss of tumor cell reliance on estrogen signaling, and that breast cancer patients with estrogen receptor-α-positive tumors expressing low levels of CDK10 relapse early on tamoxifen, which suggests that downregulation of CDK10 contributes to resistance to endocrine therapy. In that regard, DNA methylation of CDK10 was found in 18% of breast cancers, suggesting that methylation of CDK10 could be a predictive molecular marker of breast cancer sensitivity to tamoxifen.

In general, the studies cited above were carried out using a candidate gene approach, but recent progress in genome-wide methylation analysis could enable performance of unbiased methylation analyses. For example, genome-wide gene expression profiles in NCI-60 cell lines are often used to assess the association between gene expression and sensitivity to chemotherapeutic


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Table 1. Stages toward the clinical application of DNA methylation markers for prediction of sensitivity to chemotherapeutic drugs

| Gene | Function | Cancer type | Stages in clinical application | References |
|---|---|---|---|---|
| *MGMT* | DNA repair | Glioma | Several clinical studies to define sensitivity to alkylating agents have been reported. | 22,28,29 |
| *hMLH1* | Mismatch repair | Colorectal cancer | Association with sensitivity to 5-fluorouracil has been reported. Independent experiments remain to be performed. | 30 |
| *WRN* | DNA helicase | Colorectal cancer | Association with sensitivity to cisplatin has been reported. Independent experiments remain be performed. | 33 |
| *FANCF* | DNA repair | Ovarian cancer | Association with sensitivity to cisplatin has been reported. Independent experiments remain to be performed. | 34 |
| *CHFR* | Mitotic checkpoint | Colorectal cancer | Several clinical trials to define sensitivity to microtubule inhibitors have been reported. | 41,42 |
| *14-3-3σ* | $G_2$–M checkpoint | Colorectal cancer | Association with sensitivity to DNA damaging agents has been shown in cell lines. Clinical studies remain to be performed. | 47 |
| *CDK10* | $G_2$–M checkpoint | Breast cancer | Association with sensitivity to tamoxifen has been reported. Independent experiments remain to be performed. | 51 |
| *p73* | DNA damage checkpoint | Renal cancer | Association with sensitivity to cisplatin has been reported for cell lines. Clinical studies remain to be performed. | 52 |

CHFR, checkpoint with ring finger; FANCF, Fanconi anemia protein F; hMLH1, human mutL homolog 1; MGMT, $O^6$-methylguanine-DNA-methyltransferase; WRN, Werner syndrome protein.

drugs. By comparing the DNA methylation profiles for 32 genes with drug sensitivity in NCI-60 cell lines, Shen *et al.* were able to identify a correlation between *p73* methylation and sensitivity to alkylating agents.[52] p73 is a member of the p53 family and, like other p53 family members, it is involved in cell-cycle checkpoint function, apoptosis, DNA repair, and cellular differentiation.[53] The findings of Shen *et al.* suggest that methylation of p73 could be a predictive marker of sensitivity to alkylating agents.[52] Consistent with that idea, overexpression of p73 has been observed in cancers of the bladder, lung, and ovary, and is often associated with resistance to treatment with DNA-damaging agents.[54–56] In addition, knocking down p73 using siRNA reduced cellular viability after treatment with BCNU and cisplatin. The molecular mechanism by which silencing of p73 sensitizes cancer cells to alkylating agents remains unknown, however.

## Epigenetic alteration of signaling pathways and resistance to therapy

Patients with *K-ras* mutations reportedly do not respond to treatment with monoclonal anti-EGFR antibodies such as cetuximab or panitumumab.[57,58] Situated downstream of EGFR, K-ras is a key component of the RAS–MAPK pathway and is involved in mediating cell proliferation. Its mutation may enable cells to circumvent the anti-EGFR activity of cetuximab and panitumumab. That colorectal cancers with *K-ras* mutations tend to show methylation of multiple CpG islands suggests that resistance to anti-EGFR therapy in patients with *K-ras* mutations may be associated with CIMP,[59] and that DNA methylation of genes affected by CIMP may also contribute to the resistance to cetuximab or panitumumab. In fact, RASSF2, a negative effector of RAS, is silenced by DNA methylation in CIMP-positive colorectal cancers.[18,60]

## Future directions in cancer epigenomics: Genome-wide approaches

Although DNA methylation of certain genes appears to influence sensitivity to chemotherapeutic drugs, the majority of studies carried out to date were done using cell line models or only a small number of subjects (Table 1). Large-scale analyses will be necessary to confirm the utility of epigenetic information for prediction of responses to chemotherapeutic drugs. Comprehensive studies of pharmacoepigenomics in cancer await advances in genome-wide DNA methylation analyses using microarrays and next-generation sequencers.

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## References

1 Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159–70.
2 Weinstein IB, Joe A. Oncogene addiction. *Cancer Res* 2008; **68**: 3077–80.
3 Lynch TJ, Bell DW, Sordella R *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350**: 2129–39.
4 Mitsudomi T, Kosaka T, Endoh H *et al.* Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. *J Clin Oncol* 2005; **23**: 2513–20.
5 Paez JG, Janne PA, Lee JC *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–500.
6 Pardanani A, Tefferi A. Imatinib targets other than bcr/abl and their clinical relevance in myeloid disorders. *Blood* 2004; **104**: 1931–9.
7 Hayes DF, Thor AD. c-erbB-2 in breast cancer: development of a clinically useful marker. *Semin Oncol* 2002; **29**: 231–45.
8 Dan S, Tsunoda T, Kitahara O *et al.* An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines. *Cancer Res* 2002; **62**: 1139–47.
9 Potti A, Dressman HK, Bild A *et al.* Genomic signatures to guide the use of chemotherapeutics. *Nat Med* 2006; **12**: 1294–300.
10 Minna JD, Girard L, Xie Y. Tumor mRNA expression profiles predict responses to chemotherapy. *J Clin Oncol* 2007; **25**: 4329–36.
11 Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007; **128**: 683–92.
12 Kanai Y, Hirohashi S. Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state. *Carcinogenesis* 2007; **28**: 2434–42.
13 Ushijima T, Okochi-Takada E. Aberrant methylations in cancer cells: where do they come from? *Cancer Sci* 2005; **96**: 206–11.



doi: 10.1111/j.1349-7006.2009.01095.x
© 2009 Japanese Cancer Association

14 Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999; **96**: 8681–6.
15 Toyota M, Issa JP. CpG island methylator phenotypes in aging and cancer. *Semin Cancer Biol* 1999; **9**: 349–57.
16 Sato H, Suzuki H, Toyota M *et al.* Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors. *Carcinogenesis* 2007; **28**: 2459–66.
17 Suzuki H, Watkins DN, Jair KW *et al.* Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 2004; **36**: 417–22.
18 Akino K, Toyota M, Suzuki H *et al.* The Ras effector RASSF2 is a novel tumor-suppressor gene in human colorectal cancer. *Gastroenterology* 2005; **129**: 156–69.
19 Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21. *3 Nat Genet* 2000; **25**: 315–19.
20 Fukayama M, Hino R, Uozaki H. Epstein–Barr virus and gastric carcinoma: virus–host interactions leading to carcinoma. *Cancer Sci* 2008; **99**: 1726–33.
21 Maekita T, Nakazawa K, Mihara M *et al.* High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. *Clin Cancer Res* 2006; **12**: 989–95.
22 Esteller M, Garcia-Foncillas J, Andion E *et al.* Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med* 2000; **343**: 1350–4.
23 Pegg AE. Mammalian $O^6$-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res* 1990; **50**: 6119–29.
24 Esteller M, Herman JG. Generating mutations but providing chemosensitivity. the role of $O^6$-methylguanine DNA methyltransferase in human cancer. *Oncogene* 2004; **23**: 1–8.
25 Esteller M, Risques RA, Toyota M *et al.* Promoter hypermethylation of the DNA repair gene $O^6$-methylguanine-DNA methyltransferase is associated with the presence of G : C to A : T transition mutations in p53 in human colorectal tumorigenesis. *Cancer Res* 2001; **61**: 4689–92.
26 Esteller M, Toyota M, Sanchez-Cespedes M *et al.* Inactivation of the DNA repair gene $O^6$-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. *Cancer Res* 2000; **60**: 2368–71.
27 Ludlum DB. DNA alkylation by the haloethylnitrosoureas: nature of modifications produced and their enzymatic repair or removal. *Mutat Res* 1990; **233**: 117–26.
28 Brell M, Tortosa A, Verger E *et al.* Prognostic significance of $O^6$-methylguanine-DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression in anaplastic gliomas. *Clin Cancer Res* 2005; **11**: 5167–74.
29 Hegi ME, Diserens AC, Godard S *et al.* Clinical trial substantiates the predictive value of *O*-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. *Clin Cancer Res* 2004; **10**: 1871–4.
30 Carethers JM, Chauhan DP, Fink D *et al.* Mismatch repair proficiency and *in vitro* response to 5-fluorouracil. *Gastroenterology* 1999; **117**: 123–31.
31 Ricciardiello L, Ceccarelli C, Angiolini G *et al.* High thymidylate synthase expression in colorectal cancer with microsatellite instability: implications for chemotherapeutic strategies. *Clin Cancer Res* 2005; **11**: 4234–40.
32 Arnold CN, Goel A, Boland CR. Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. *Int J Cancer* 2003; **106**: 66–73.
33 Agrelo R, Cheng WH, Setien F *et al.* Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. *Proc Natl Acad Sci USA* 2006; **103**: 8822–7.
34 Taniguchi T, Tischkowitz M, Ameziane N *et al.* Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. *Nat Med* 2003; **9**: 568–74.
35 Marsit CJ, Liu M, Nelson HH, Posner M, Suzuki M, Kelsey KT. Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival. *Oncogene* 2004; **23**: 1000–4.
36 Narayan G, Arias-Pulido H, Nandula SV *et al.* Promoter hypermethylation of FANCF: disruption of Fanconi Anemia–BRCA pathway in cervical cancer. *Cancer Res* 2004; **64**: 2994–7.
37 Cheung HW, Jin DY, Ling MT *et al.* Mitotic arrest deficient 2 expression induces chemosensitization to a DNA-damaging agent, cisplatin, in nasopharyngeal carcinoma cells. *Cancer Res* 2005; **65**: 1450–8.
38 Wang X, Jin DY, Wong HL, Feng H, Wong YC, Tsao SW. MAD2-induced sensitization to vincristine is associated with mitotic arrest and Raf/Bcl-2 phosphorylation in nasopharyngeal carcinoma cells. *Oncogene* 2003; **22**: 109–16.
39 Anand S, Penrhyn-Lowe S, Venkitaraman AR. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell* 2003; **3**: 51–62.
40 Satoh A, Toyota M, Itoh F *et al.* Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. *Cancer Res* 2003; **63**: 8606–13.
41 Koga Y, Kitajima Y, Miyoshi A, Sato K, Sato S, Miyazaki K. The significance of aberrant CHFR methylation for clinical response to microtubule inhibitors in gastric cancer. *J Gastroenterol* 2006; **41**: 133–9.
42 Ogi K, Toyota M, Mita H *et al.* Small interfering RNA-induced CHFR silencing sensitizes oral squamous cell cancer cells to microtubule inhibitors. *Cancer Biol Ther* 2005; **4**: 773–80.
43 Toyota M, Sasaki Y, Satoh A *et al.* Epigenetic inactivation of CHFR in human tumors. *Proc Natl Acad Sci USA* 2003; **100**: 7818–23.
44 Sun Y. Targeting E3 ubiquitin ligases for cancer therapy. *Cancer Biol Ther* 2003; **2**: 623–9.
45 Hermeking H, Lengauer C, Polyak K *et al.* 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* 1997; **1**: 3–11.
46 Ferguson AT, Evron E, Umbricht CB *et al.* High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proc Natl Acad Sci USA* 2000; **97**: 6049–54.
47 Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Imai K. Inactivation of the 14-3-3 sigma gene is associated with 5′-CpG island hypermethylation in human cancers. *Cancer Res* 2000; **60**: 4353–7.
48 Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 1999; **401**: 616–20.
49 Chan TA, Hwang PM, Hermeking H, Kinzler KW, Vogelstein B. Cooperative effects of genes controlling the $G_2$/M checkpoint. *Genes Dev* 2000; **14**: 1584–8.
50 Liu Y, Liu H, Han B, Zhang JT. Identification of 14-3-3sigma as a contributor to drug resistance in human breast cancer cells using functional proteomic analysis. *Cancer Res* 2006; **66**: 3248–55.
51 Iorns E, Turner NC, Elliott R *et al.* Identification of CDK10 as an important determinant of resistance to endocrine therapy for breast cancer. *Cancer Cell* 2008; **13**: 91–104.
52 Shen L, Toyota M, Kondo Y *et al.* Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc Natl Acad Sci USA* 2007; **104**: 18 654–9.
53 Ozaki T, Nakagawara A. p73, a sophisticated p53 family member in the cancer world. *Cancer Sci* 2005; **96**: 729–37.
54 Nyman U, Sobczak-Pluta A, Vlachos P, Perlmann T, Zhivotovsky B, Joseph B. Full-length p73α represses drug-induced apoptosis in small cell lung carcinoma cells. *J Biol Chem* 2005; **280**: 34 159–69.
55 Vikhanskaya F, Marchini S, Marabese M, Galliera E, Broggini M. P73a overexpression is associated with resistance to treatment with DNA-damaging agents in a human ovarian cancer cell line. *Cancer Res* 2001; **61**: 935–8.
56 Yokomizo A, Mai M, Tindall DJ *et al.* Overexpression of the wild type p73 gene in human bladder cancer. *Oncogene* 1999; **18**: 1629–33.
57 Freeman DJ, Juan T, Reiner M *et al.* Association of K-ras mutational status and clinical outcomes in patients with metastatic colorectal cancer receiving panitumumab alone. *Clin Colorectal Cancer* 2008; **7**: 184–90.
58 Khambata-Ford S, Garrett CR, Meropol NJ *et al.* Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol* 2007; **25**: 3230–7.
59 Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc Natl Acad Sci USA* 2000; **97**: 710–15.
60 Kakar S, Deng G, Cun L, Sahai V, Kim YS. CpG island methylation is frequently present in tubulovillous and villous adenomas and correlates with size, site, and villous component. *Hum Pathol* 2008; **39**: 30–6.


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# The Epigenome of Colorectal Cancer

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Epigenetic alterations (eg, DNA methylation) play important roles in silencing cancer-related genes in colorectal cancers (CRCs). DNA methylation occurs in genes involved in cell cycle checkpoints, apoptosis, signal transduction, DNA repair, and maintenance of the genome's integrity. Recent developments of new methods for detecting DNA methylation have enabled us to create epigenetic profiles of CRC and to classify them into three distinct subgroups based on genetic and epigenetic alterations. DNA methylation also leads to silencing of some microRNAs, which in turn leads to dysregulation of oncogenic proteins, which are their targets. Moreover, for diagnosis, epigenetic information may be used to detect cancer cells in serum and stool. Obtaining a fuller understanding of the epigenome will be an important step toward understanding the molecular mechanisms underlying CRC and may provide the basis for the development of novel diagnostic tools and approaches to therapy.

## Introduction

Colorectal cancer (CRC) arises through the accumulation of genetic changes, including mutation, amplification, and deletion of genes. Identification of these changes (eg, underlying familial cancer syndromes, such as familial adenomatous polyposis and hereditary nonpolyposis CRC [HNPCC]) has shed light on the disease process by providing information about the contributions to colorectal tumorigenesis made by Wnt signaling and impairment of mismatch repair genes [1]. Genome-wide screening has revealed that CRCs are associated with numerous gene mutations [2]. Integrated analysis of homozygous deletions and focal gene amplification have identified at least 17 altered genes per tumor, although the frequency of these mutations are low, with the exception of *APC*, *KRAS*, and p53 [3].

In contrast to genetic alterations, epigenetic alterations (eg, DNA methylation and modification of histone tails) have been only recently extensively studied. This is primarily because there was no procedure to examine DNA methylation, and it was not known which genes should be tested. However, progress in the methodology for detecting DNA methylation has significantly improved this situation. In this review, we provide an overview of recent progress in analysis of the epigenome in CRC.

## Epigenetic Regulation of Gene Expression

*Epigenetics* refers to heritable modifications of DNA that do not affect the nucleotide sequence. DNA methylation and modification of histone are the best studied among such phenomena. Under normal physiologic conditions, DNA methylation (catalyzed by three DNA methyltransferases [DNMT1, DNMT3A, and DNMT3B]), plays an important role in gene imprinting, X chromosome inactivation, and silencing of repetitive sequences. Underlying these effects are significant methylation-induced changes in the chromatin structure, including recruitment of methyl-CpG binding domain proteins and deacetylation and methylation of histone tails. Notably, knockout of DNMT1 and DNMT3B in the HCT116 CRC cell line (double-knockout cells) results in demethylation, leading to a 95% loss of methylcytosines [4]. When CRC cells are treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) with the histone deacetylase inhibitor trichostatin, the two act synergistically to induce gene expression [5]. Moreover, analysis of genes targeted by polycomb-repressive complexes (PRCs) in pluripotent embryonic stem cells showed that patterns of polycomb-based repression are closely associated with targets of DNA methylation in cancer, suggesting there is cross-talk between polycomb targeting and DNA methylation [6,7]. Consistent with that idea, EZH2, a histone methyltransferase component of PRC2, is frequently overexpressed in cancer [8].

## Mapping DNA Methylation in the Human Genome

One of the obstacles to studying epigenetics in cancer has been the absence of a procedure for analyzing DNA

**Table 1. Methods for detecting DNA methylation changes in colorectal cancer**

| Study | Methods | Principles of technologies |
|---|---|---|
| | **Methylation detection** | |
| Eads et al. [9] | Methylation-specific PCR | Bisulfite conversion and allele-specific PCR |
| Herman et al. [10] | MethyLight | Bisulfite conversion and real-time PCR |
| Clark et al. [11] | Bisulfite sequencing | Bisulfite conversion, PCR, and sequencing |
| Xiong and Laird [12] | COBRA | Bisulfite conversion, PCR, and restriction digestion |
| Uhlmann et al. [13] | Bisulfite pyrosequencing | Bisulfite conversion, PCR, and pyrosequencing |
| | **Methylation screening** | |
| Costello et al. [14] | RLGS | Restriction digestion, electrophoresis |
| Ushijima et al. [15] | MS-RDA | Restriction digestion, adaptor ligation, and PCR |
| Toyota et al. [16] | MCA-RDA | Restriction digestion, adaptor ligation, and PCR |
| Estecio et al. [17] | MCA-array | Restriction digestion, adaptor ligation, PCR, and microarray |

COBRA—combined bisulfite restriction analysis; MCA-array—methylated CpG island amplification–array; MCA-RDA—methylated CpG island amplification restriction difference analysis; MS-RDA—methylation-sensitive representational difference analysis; PCR—polymerase chain reaction; RLGS—restriction landmark genomic scanning.

methylation. Restriction enzyme digestion followed by Southern blotting has been used, but this method requires high-quality DNA and is time consuming. However, the development of bisulfate-based methylation analysis has dramatically improved the study of DNA methylation (Table 1). With this approach, allele-specific polymerase chain reaction can be used to detect methylated and unmethylated alleles [9,10], after which the amplified products can be sequenced [11] or digested using restriction enzymes that selectively recognize sites containing a CpG site [12]. By applying pyrosequencing, DNA methylation levels can be determined more precisely [13].

Another factor that has limited the study of DNA methylation in cancer is the lack of markers to study. Several approaches enable differential screening of methylated genes in cancer and normal tissues (Table 1). Restriction landmark genomic scanning is one technique. With more than 1000 genes analyzed in each experiment, restriction landmark genomic scanning can be used to evaluate methylation throughout the genome and can be applied to detect cancer-related changes in DNA methylation [14]. Methylation-sensitive representational analysis is another technique that was developed to detect differences between DNA methylation in cancer and normal tissues [15]. However, a limitation of this approach is that methylated alleles are detected as a loss of signal, reflecting the inability of methylation-sensitive restriction enzymes to digest at CpG sites.

We have developed a method called *methylated CpG island amplification* (MCA). MCA enables us to amplify only methylated sequences. DNA fragments differentially methylated in cancer and normal tissues can be identified using an MCA amplicon as a tester and driver to carry out representational difference analysis [16]. MCA can also be applied to promoter microarrays to identify genes methylated in cancer but not in normal tissue [17].

## Role of DNA Methylation in Tumorigenesis of CRC

Earlier studies of DNA methylation demonstrated that the calcitonin gene is hypermethylated in CRC [18]. It was unclear whether methylation of genes such as calcitonin was really involved in tumorigenesis or whether it was an epiphenomenon. However, recent studies have confirmed that DNA methylation plays an important role in tumorigenesis in the colon. For example, it was shown that DNA methylation affects genes such as *RB*, *VHL*, *APC*, *CDH1*, and *BRCA1*, all of which are involved in familial cancer syndrome [19]. Additionally, DNA methylation serves as one of two hit mechanisms for gene inactivation, and genes infrequently mutated in CRCs are often targets of DNA methylation. For example, the *CDKN2A/p16* gene is rarely mutated or deleted in CRC, but it is inactivated by methylation in 40% of tumors [20]. *CDKN2A/p16* methylation is also seen in colorectal adenomas, indicating that DNA methylation is already present in premalignant lesions.

Genes involved in signal transduction pathways are often targets of DNA methylation, among which we identified the secreted frizzled-related protein (SFRP) gene family as one such target [21]. Their products, SFRPs, antagonize Wnt signaling and are frequently silenced in CRC [22]. Ectopic expression of SFRPs suppresses T-cell factor/β-catenin activity in CRC cells carrying *APC* or β-catenin mutations and other negative regulators of Wnt signaling, such as *DKK* genes, which are also silenced by DNA methylation in CRC [23]. This suggests that not only mutations of *APC*/β-catenin, but

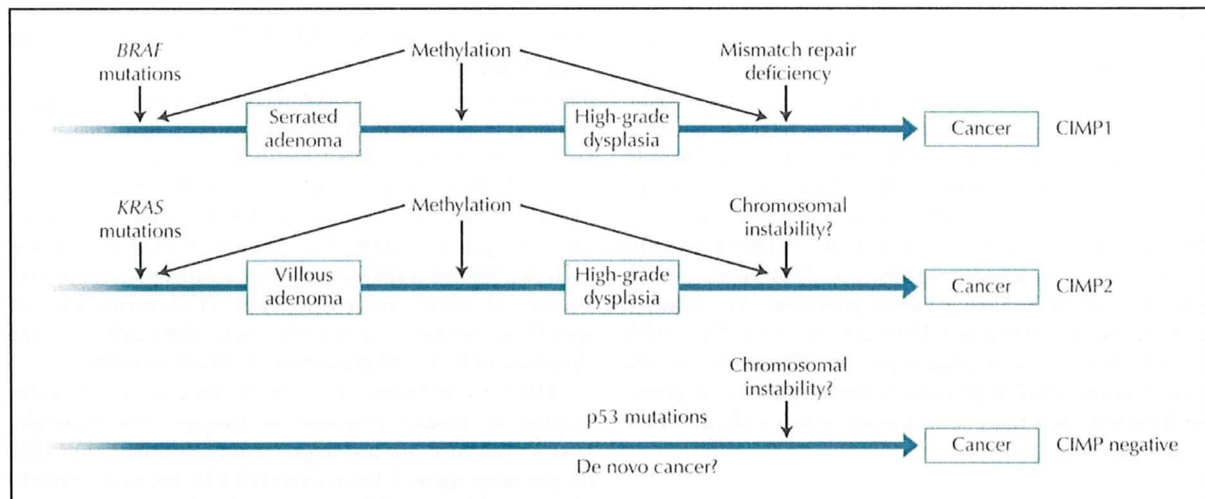


**Figure 1.** Colorectal cancers can be grouped into three distinct groups. CpG island methylator phenotype 1 (CIMP1) tumors, which have a high degree of DNA methylation, frequently show microsatellite instability because of methylation of *hMLH1* and *BRAF* mutation. It has been suggested that CIMP1 tumors arise through the sessile serrated adenoma pathway. CpG island methylator phenotype 2 (CIMP2) tumors frequently show mutation of *KRAS*, but the frequencies of microsatellite instability, *BRAF*, and p53 mutations are low. These tumors have the poorest prognosis. CIMP-negative tumors frequently show p53 mutations.

epigenetic inactivation of negative regulators of Wnt is important for full activation of Wnt signaling during tumorigenesis in CRC.

Mutation of *KRAS* is frequently detected in CRCs and adenomas. In normal cells, activation of *KRAS* induces senescence and apoptosis mediated by negative regulators of Ras [24]. Our group and others have found that two of these negative regulators, Ras association domain family proteins 1 and 2 (RASSF1 and RASSF2), are methylated in CRCs, and ectopic expression of RASSF2 induces morphologic changes in cells and apoptosis [25,26]. Moreover, knockdown of RASSF2 enhances *KRAS*-mediated cellular transformation, indicating RASSF2 has the ability to prevent cellular transformation. Primary CRCs that show *KRAS/BRAF* mutations also frequently show RASSF2 methylation, and the resultant inactivation of RASSF2 enhances *KRAS*-induced oncogenic transformation. DNA methylation also occurs in genes involved in DNA repair and maintaining the integrity of the genome (eg, *MGMT, WRN* [27,28]), inhibition of angiogenesis (eg, *THBS1* [29]), and tumor immunity (eg, *CIITA* [30]).

## CpG Island Methylator Phenotype and Microsatellite Instability

HNPCC shows microsatellite instability (MSI), which leads to alteration of genes containing microsatellite tracts. In HNPCC, MSI is caused by mutations of mismatch repair genes, such as *hMSH2* and *hMLH1* [31]. About 15% to 20% of sporadic CRCs show MSI, although mutation of mismatch repair genes is not frequent, and one mismatch repair gene, *hMLH*, is inactivated by DNA methylation [32]. During the course of our work profiling methylation in CRC, we found that a subset of cancers shows the CpG island methylator phenotype (CIMP), and methylation of *hMLH1* correlates significantly with CIMP, suggesting CIMP causes MSI, which in turn leads to inactivation of *hMLH1* [33].

## Distinct Genetic and Epigenetic Alterations in Three Types of CRC

Until recently, the existence of CIMP was controversial because there were no optimal markers to define it. This changed when Weisenberger et al. [34] were able to identify CIMP in CRC using five markers detected using MethyLight. However, these investigators likely focused solely on CIMP1 cases and thus underestimated the CIMP2 group. Recently, Shen et al. [35] analyzed mutations of *BRAF, KRAS*, and p53 and methylation of 27 loci in 97 CRCs; they found that the tumors could be grouped based on the genetic and epigenetic alterations. Unsupervised hierarchical clustering of the DNA methylation data identified three distinct groups of colon cancers: CIMP1, CIMP2, and CIMP negative (Fig. 1). CIMP1 cases showed a high frequency of MSI and *BRAF* mutations (80% and 53%, respectively) but few instances of *KRAS* and/or p53 mutations (16% and 11%, respectively). Conversely, CIMP2 cases were associated with a high frequency of *KRAS* mutations (92%), but MSI and *BRAF* mutations rarely occurred (0% and 4%, respectively), and there was only a low rate of p53 mutations (31%). CIMP-negative cases had a higher rate of p53 mutations (71%) and a lower rate of MSI (12%) and

mutations of *BRAF* (2%) and *KRAS* (33%). The existence of three groups of CRCs was confirmed in a larger study by Barault et al. [36]; they assessed 582 cases. Additionally, there was an inverse correlation between chromosomal instability and the presence of CIMP, indicating that CIMP-positive CRCs have distinct genetic and epigenetic features. Most importantly, CIMP affects the prognosis of patients with CRC. CIMP1 patients have a relatively good prognosis, as MSI-positive CRC patients generally have a better prognosis. By contrast, patients with CIMP2 or CIMP-low microsatellite-stable tumors have a poor prognosis [36,37]. However, the existence of CIMP-high tumors without *BRAF* mutation or MSI indicates there is still more to learn about CIMP and its classification.

## Epigenetic Alteration of MicroRNA Expression

MicroRNAs (miRNAs) are a group of small noncoding RNAs that negatively regulate the translation and stability of partially complementary target mRNAs [38]. Downregulation of a subset of miRNAs is a commonly observed feature of cancers, suggesting these molecules may act as tumor suppressors [39,40]. By identifying miRNAs whose expression was upregulated in CRC cells in which DNMT1 and DNMT3B were knocked out, we were able to identify miRNAs that were epigenetically silenced [41]. Among the 37 miRNAs upregulated by DNMT inhibition, we focused on *miR-34b/c* because recent studies have shown that *miR-34* family members (*miR-34a, miR-34b*, and *miR-34c*) are direct targets of p53 [40,42,43]. We found that downregulation of *miR-34b/c* expression was strongly associated with methylation of its neighboring CpG island, which harbors bidirectional promoter activity and also regulates expression of another candidate tumor suppressor gene, B-cell translocation gene 4 (*BTG4*). In addition, ectopic expression of the *miR-34b/c* precursor in CRC cells resulted in downregulation of CDK6, a cyclin-dependent kinase involved in cell growth, and MET, a receptor tyrosine kinase involved in cell growth and metastasis. Epigenetic inactivation of *miR-34b/c* may then attenuate p53 function through dysregulation of the cell cycle and cell growth.

Lujambio et al. [44] showed that *miR-124a*, another regulator of CDK6, is targeted for epigenetic inactivation by methylation in 75% of CRCs. Transfection of *miR-124a* into colon cancer cell lines suppressed levels of CDK6 protein, which led to reduced phosphorylation of ribose in residues 807 and 811. More recently, Lujambio et al. [45] extended their analysis to identify *miR-9*, *miR-34b/c*, and *miR-148a* as targets of epigenetic inactivation in metastatic CRC, which suggests that epigenetic inactivation plays a key role in the silencing of protein-coding genes and noncoding RNA.

## Implications of DNA Methylation in Diagnosis and Therapy

The tumor specificity of DNA methylation profiles suggests it should be possible to diagnose cancers on that basis. Consistent with that idea, DNA methylation can be detected in the fecal DNA of colon cancer patients [46] and the serum of CRC patients [47]. Moreover, the fact that methylation of certain genes (eg, *SFRP1*) can be detected in early lesions, such as aberrant crypt foci, suggests methylation levels may be used to assess cancer risk. Thus, DNA methylation of specific genes may serve as useful molecular markers for the diagnosis of CRC and prediction of patient outcome.

DNA methylation also can be used as a molecular marker to predict response to therapy. For example, CRCs with MSI are less aggressive than others, but they do not respond to 5-fluorouracil (5-FU) because of methylation of *hMLH1* [48]. However, they become responsive upon exposure to 5-aza-2′-deoxycytidine (5-aza-dC) [49]. Thus, methylation of *hMLH1* appears to be a predictive molecular marker of the sensitivity of CRCs to 5-FU. Similarly, methylation of *WRN* correlates with the sensitivity of CRC cells to topoisomerase inhibitors [27], whereas methylation of *MGMT* and *p73* correlates with sensitivity to alkylating agents [50••], and methylation of *CHFR* (a mitotic checkpoint gene) correlates with sensitivity to microtubule inhibitors [51].

Because epigenetic changes are reversible, DNA methylation and histone modification can be reversed using DNA methyltransferase inhibitors or histone deacetylase inhibitors, and DNA methyltransferase inhibitors (azacytidine and decitabine) have been used to treat patients with myelodysplastic syndrome [52]. Although such epigenetic therapy has not yet proved effective in the treatment of CRC, it can be combined with chemotherapeutic agents to enhance the effects of those drugs. For instance, CRCs escape the immune system through inactivation of the tumor antigen presentation system. Treating cancer cells with DNA methyltransferase inhibitors or histone deacetylase inhibitors restores tumor antigen, thereby stimulating immune responses.

## Conclusions

We anticipate that a great deal of new information about the role of the epigenome in CRC will become available in the near future. Understanding the epigenome will be an important step toward a fuller understanding of the molecular mechanisms underlying CRC and may provide the basis for the development of novel diagnostic methods and approaches to therapy.

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## Disclosure

No potential conflicts of interest relevant to this article were reported.

## References and Recommended Reading

Papers of particular interest, published recently, have been highlighted as:

- • Of importance
- •• Of major importance


1. Kinzler KW, Vogelstein B: Lessons from hereditary colorectal cancer. *Cell* 1996, 87:159–170.
2. Wood LD, Parsons DW, Jones S, et al.: The genomic landscapes of human breast and colorectal cancers. *Science* 2007, 318:1108–1113.
3. Leary RJ, Lin JC, Cummins J, et al.: Integrated analysis of homozygous deletions, focal amplifications, and sequence alterations in breast and colorectal cancers. *Proc Natl Acad Sci U S A* 2008, 105:16224–16229.
4. Rhee I, Bachman KE, Park BH, et al.: DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 2002, 416:552–556.
5. Cameron EE, Bachman KE, Myohanen S, et al.: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 1999, 21:103–107.
6. Ohm JE, McGarvey KM, Yu X, et al.: A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet* 2007, 39:237–242.
7. Widschwendter M, Fiegl H, Egle D, et al.: Epigenetic stem cell signature in cancer. *Nat Genet* 2007, 39:157–158.
8. Watanabe Y, Toyota M, Kondo Y, et al.: PRDM5 identified as a target of epigenetic silencing in colorectal and gastric cancer. *Clin Cancer Res* 2007, 13:4786–4794.
9. Eads CA, Danenberg KD, Kawakami K, et al.: MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res* 2000, 28:E32.
10. Herman JG, Graff JR, Myohanen S, et al.: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996, 93:9821–9826.
11. Clark SJ, Harrison J, Paul CL, Frommer M: High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 1994, 22:2990–2997.
12. Xiong Z, Laird PW: COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res* 1997, 25:2532–2534.
13. Uhlmann K, Brinckmann A, Toliat MR, et al.: Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. *Electrophoresis* 2002, 23:4072–4079.
14. Costello JF, Fruhwald MC, Smiraglia DJ, et al.: Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* 2000, 24:132–138.
15. Ushijima T, Morimura K, Hosoya Y, et al.: Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. *Proc Natl Acad Sci U S A* 1997, 94:2284–2289.
16. Toyota M, Ho C, Ahuja N, et al.: Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res* 1999, 59:2307–2312.
17. Estecio MR, Yan PS, Ibrahim AE, et al.: High-throughput methylation profiling by MCA coupled to CpG island microarray. *Genome Res* 2007, 17:1529–1536.
18. Silverman AL, Park JG, Hamilton SR, et al.: Abnormal methylation of the calcitonin gene in human colonic neoplasms. *Cancer Res* 1989, 49:3468–3473.
19. Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002, 3:415–428.
20. Herman JG, Merlo A, Mao L, et al.: Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res* 1995, 55:4525–4530.
21. Suzuki H, Watkins DN, Jair KW, et al.: Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 2004, 36:417–422.
22. Bovolenta P, Esteve P, Ruiz JM, et al.: Beyond Wnt inhibition: new functions of secreted frizzled-related proteins in development and disease. *J Cell Sci* 2008, 121:737–746.
23. Sato H, Suzuki H, Toyota M, et al.: Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors. *Carcinogenesis* 2007, 28:2459–2466.
24. Benanti JA, Galloway DA: The normal response to RAS: senescence or transformation? *Cell Cycle* 2004, 3:715–717.
25. Akino K, Toyota M, Suzuki H, et al.: The Ras effector RASSF2 is a novel tumor-suppressor gene in human colorectal cancer. *Gastroenterology* 2005, 129:156–169.
26. van Engeland M, Roemen GM, Brink M, et al.: K-ras mutations and RASSF1A promoter methylation in colorectal cancer. *Oncogene* 2002, 21:3792–3795.
27. Agrelo R, Cheng WH, Setien F, et al.: Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. *Proc Natl Acad Sci U S A* 2006, 103:8822–8827.
28. Esteller M, Hamilton SR, Burger PC, et al.: Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 1999, 59:793–797.
29. Ahuja N, Mohan AL, Li Q, et al.: Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res* 1997, 57:3370–3374.
30. Satoh A, Toyota M, Ikeda H, et al.: Epigenetic inactivation of class II transactivator (CIITA) is associated with the absence of interferon-gamma-induced HLA-DR expression in colorectal and gastric cancer cells. *Oncogene* 2004, 23:8876–8886.
31. Chung DC, Rustgi AK: DNA mismatch repair and cancer. *Gastroenterology* 1995, 109:1685–1699.
32. Cunningham JM, Christensen ER, Tester DJ, et al.: Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res* 1998, 58:3455–3460.
33. Toyota M, Ahuja N, Ohe-Toyota M, et al.: CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 1999, 96:8681–8686.
34. Weisenberger DJ, Siegmund KD, Campan M, et al.: CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006, 38:787–793.
35. Shen L, Toyota M, Kondo Y, et al.: Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc Natl Acad Sci U S A* 2007, 104:18654–18659.

36. Barault L, Charon-Barra C, Jooste V, et al.: **Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases.** *Cancer Res* 2008, 68:8541–8546.
37. Shen L, Catalano PJ, Benson AB 3rd, et al.: **Association between DNA methylation and shortened survival in patients with advanced colorectal cancer treated with 5-fluorouracil based chemotherapy.** *Clin Cancer Res* 2007, 13:6093–6098.
38. He L, Hannon GJ: **MicroRNAs: small RNAs with a big role in gene regulation.** *Nat Rev Genet* 2004, 5:522–531.
39. He X, He L, Hannon GJ: **The guardian's little helper: microRNAs in the p53 tumor suppressor network.** *Cancer Res* 2007, 67:11099–11101.
40. Hermeking H: **p53 enters the microRNA world.** *Cancer Cell* 2007, 12:414–418.
41. Toyota M, Suzuki H, Sasaki Y, et al.: **Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer.** *Cancer Res* 2008, 68:4123–4132.
42. Bommer GT, Gerin I, Feng Y, et al.: **p53-mediated activation of miRNA34 candidate tumor-suppressor genes.** *Curr Biol* 2007, 17:1298–1307.
43. He L, He X, Lim LP, et al.: **A microRNA component of the p53 tumour suppressor network.** *Nature* 2007, 447:1130–1134.
44. Lujambio A, Ropero S, Ballestar E, et al.: **Genetic unmasking of an epigenetically silenced microRNA in human cancer cells.** *Cancer Res* 2007, 67:1424–1429. [Published erratum appears in *Cancer Res* 2007, 67:3492.]
45. Lujambio A, Calin GA, Villanueva A, et al.: **A microRNA DNA methylation signature for human cancer metastasis.** *Proc Natl Acad Sci U S A* 2008, 105:13556–13561.
46. Chen WD, Han ZJ, Skoletsky J, et al.: **Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene.** *J Natl Cancer Inst* 2005, 97:1124–1132.
47. Wallner M, Herbst A, Behrens A, et al.: **Methylation of serum DNA is an independent prognostic marker in colorectal cancer.** *Clin Cancer Res* 2006, 12:7347–7352.
48. Carethers JM, Chauhan DP, Fink D, et al.: **Mismatch repair proficiency and in vitro response to 5-fluorouracil.** *Gastroenterology* 1999, 117:123–131.
49. Arnold CN, Goel A, Boland CR: **Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines.** *Int J Cancer* 2003, 106:66–73.
50.•• Shen L, Kondo Y, Ahmed S, et al.: **Drug sensitivity prediction by CpG island methylation profile in the NCI-60 cancer cell line panel.** *Cancer Res* 2007, 67:11335–11343.

This is a comprehensive analysis examining the relationship between DNA methylation and sensitivity to chemotherapeutic drugs.

51. Toyota M, Sasaki Y, Satoh A, et al.: **Epigenetic inactivation of CHFR in human tumors.** *Proc Natl Acad Sci U S A* 2003, 100:7818–7823.
52. Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H: **Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies.** *Cancer* 2008, 112:2341–2351.

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# Integrated analysis of genetic and epigenetic alterations in cancer

A proposed genetic model describing the transition from normal colonic epithelium to malignant cancer involves mutation of a number of key oncogenes and tumor suppressor genes. However, only subsets of colorectal cancers contain such mutations. Moreover, the heterogeneous pattern of tumor mutations suggests there are multiple alternative pathways leading to colonic tumorigenesis. These alternative pathways involve epigenetic alterations such as the methylation of multiple CpG islands, termed the CpG island methylator phenotype, and cancers with CpG island methylator phenotype show distinct genetic and clinicopathological features. The causes of these epigenetic alterations are still not fully understood, but exogenous pathogens such as *Helicobacter pylori* and Epstein–Barr virus, and the chromosomal translocations seen in leukemia, have all been shown to induce epigenetic alterations of genes.

**KEYWORDS:** CpG island methylator phenotype colorectal cancer DNA methylation epigenetics histone tumor suppressor gene

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It is now well established that both genetic and epigenetic alterations accumulate during carcinogenesis. Among the epigenetic changes identified to date, DNA methylation, particularly methylation of the 5′ CpG islands of genes, is the best characterized. Under normal physiological conditions, DNA methylation catalyzed by three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) is involved in regulating genome imprinting, X-chromosome inactivation and inactivation of repetitive sequences [1]. In addition to DNA methylation, acetylation and methylation of lysine residues in histones H3 and H4 also play key roles in gene regulation [2]. In general, increases in histone acetylation are associated with increases in transcriptional activity, while decreases in acetylation are associated with gene repression (FIGURE 1). The acetylation status of histones reflects the balance between the activities of histone acetyltransferase and histone deacetylase. Histones H3 and H4 are also modified by methylation: methylation of H3 lysine 4 is associated with active transcription, while methylation of H3 lysines 9 and 27 is associated with gene repression (FIGURE 1). Histone modification is also mediated by the polycomb group proteins, which are negative regulators of transcription that act by forming multiple polycomb-repressive complexes (PRCs). Among the PRCs, PRC2, which contains three core components, EZH2, SUZ12 and EED, plays a key role in gene silencing in cancer. EZH2 exhibits histone methyltransferase activity, and SUZ12 and EED are required for that activity (FIGURE 2). Analysis of genes targeted by PRC2 in pluripotent embryonic stem cells showed that the patterns of polycomb-based repression in cancer are closely associated with the targets of DNA methylation, suggesting that there is a cross-talk between DNA methylation and the targeting of PRC2 [3,4]. Notably, DNA methylation affects a number of tumor suppressor genes, including *RB*, *VHL*, *BRCA1* and *CDH1* [2], as well as genes involved in cell-cycle checkpoints, apoptosis and inhibition of angiogenesis [5]. In the current review, we focus on the association between genetic and epigenetic alterations in human cancer. We also discuss recent progress in genome-wide analysis of DNA methylation.

## Genetic & epigenetic interactions in colorectal cancer

Cancer-related genetic and epigenetic alterations are perhaps best characterized in colorectal cancer. A genetic model describing the transition from normal colonic epithelium to increasingly dysplastic adenoma and then to malignant cancer has been proposed in which a number of key oncogenes and tumor suppressor genes are identified [6]. Among these is *APC*, which was first identified as a gene responsible for familial adenomatous polyposis [7]. Inactivation of *APC* leads to activation of WNT signaling through translocation of β-catenin to the nucleus [8]. In addition, *K-ras* mutation, which has been shown to be an early event in carcinogenesis and to result in the constitutive activation of Ras signaling, is detected in 30–50% of colorectal cancers.

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Finally, *p53*, which regulates a variety of genes involved in cell-cycle checkpoints, apoptosis, angiogenesis inhibition and immune responses, is one of the most frequently altered tumor suppressor genes in human cancers. However, several studies have suggested that the classic model of colorectal cancer evolution will only rationalize tumor formation in a small fraction of colorectal tumors. For instance, Smith *et al.* reported that only 6% of colorectal cancers contain mutations of *APC*, *K-ras* and *p53* [9], and the heterogeneous pattern of tumor mutations suggests there are likely multiple alternative pathways leading to colorectal cancer. A subset of familial colorectal cancers, called hereditary nonpolyposis colorectal cancer, results from germline mutations in mismatch repair (MMR) genes [6]. Microsatellite sequences, such as mono- and di-nucleotide repeats, are frequently mutated because of this MMR deficiency. In addition, approximately 15–20% of sporadic colorectal cancers show microsatellite instability (MSI), which is caused by the lack of MMR gene expression. MMR mutations are rarely found in these tumors, however [10].

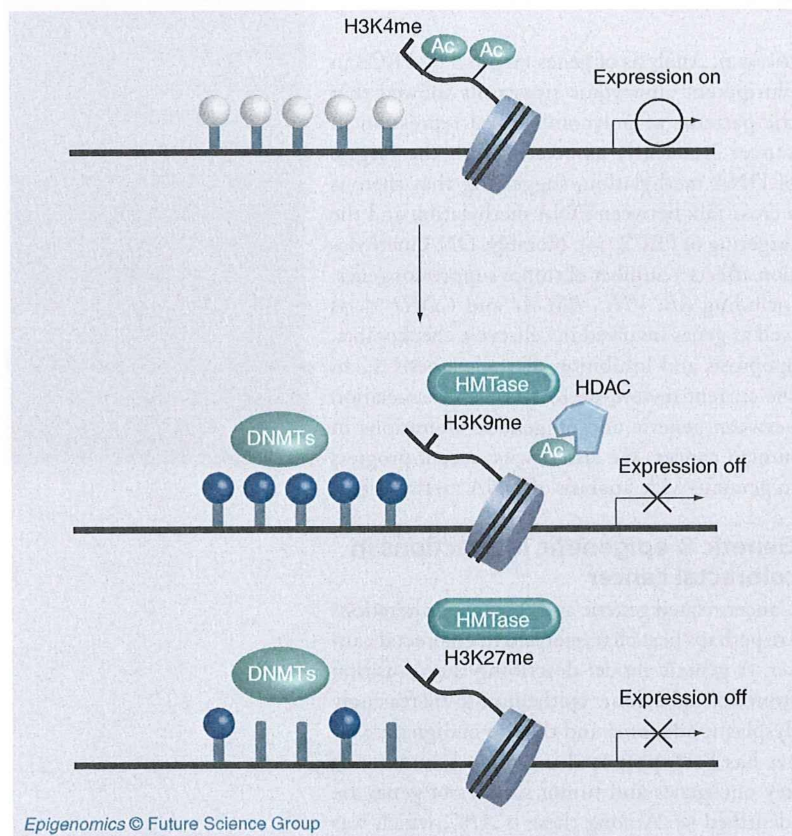


**Figure 1. The role of DNA methylation and histone modification in gene expression.** In the active promoter, CpG sites are unmethylated, most of the lysine residues in the histone tail are acetylated (Ac), and histone H3 lysine 4 is methylated. In a repressed promoter, DNMTs methylate CpG sites, HDAC removes acetyl groups, and histone H3 lysines 9 and 27 are modified by HMTase. Gray circles: Unmethylated CpG sites; blue circles: Methylated CpG sites. DNMT: DNA methyltransferase; HDAC: Histone deacetylase; HMTase: Histone methyltransferase.

Although one early study showed that hypomethylation of DNA is a hallmark of colorectal cancer [11], changes in DNA methylation in cancer were not very well studied until methylation of the cell-cycle checkpoint gene *CDKN2A/p16* was reported [12]. Subsequent studies simultaneously showed that the angiogenesis inhibitor *TSP1/THBS1* [13] and the DNA repair genes *hMLH1* [14] and *MGMT* [15] are all inactivated by DNA methylation. In the early days of epigenetic research it was thought that DNA methylation likely accumulated as a result of selection, but DNA methylation often silences oncogenes such as *COX2*, *TERT* and *EGFR* [16–18]. As such epigenetic silencing of oncogenes could confer a growth disadvantage to cancer cells, a simple Darwinian selection model may not be applicable to changes in DNA methylation in cancer. In addition, sporadic colorectal cancer with MSI showed methylation of multiple CpG islands [13], which suggests that CpG island methylation is not a random occurrence.

The development of techniques with which to screen for DNA methylation – for example, methylated CpG island amplification (MCA) – has enabled us to analyze the methylation of multiple genes [19]. Using markers identified by MCA, we found a subset of colorectal cancers that showed methylation of multiple CpG islands – that is, the CpG island methylator phenotype (CIMP) [20]. Furthermore, there was a significant association between hMLH1 methylation and CIMP, suggesting that MSI-positive colorectal cancer may be caused by CIMP [20]. To investigate the interaction between genetic and epigenetic factors in colorectal cancer, we first examined the rates of *K-ras* and *p53* mutation in tumors with and without CIMP. We found that CIMP cancers were characterized by a high frequency of *K-ras* mutations and a low frequency of *p53* mutations [21]. Moreover, the interactions between CIMP and *K-ras* and *p53* mutations were preserved in colorectal adenomas, suggesting that they occur early during carcinogenesis [21]. Recently, Shen *et al.* reported that colorectal cancers can be divided into three distinct subgroups based on differences in their CIMP status (Figure 3, [22]): CIMP1 cases have a high frequency of MSI and *BRAF* mutations; CIMP2 cases have with a high frequency of *K-ras* mutations, but not

MSI or *BRAF* mutations; and CIMP-negative cases have a higher frequency of p53 mutations, but not MSI, *K-ras* or *BRAF* mutations.

A growing number of studies have now confirmed the existence of CIMP and described its unique disease characteristics [23,24]. CIMP-positive cancers tend to occur in older patients, in proximal locations and in female patients [23]. Colorectal cancers with different CIMP statuses appear to have distinct precancerous lesions, including hyperplastic polyps and serrated adenomas for CIMP1 and villous adenomas for CIMP2 [25,26]. CIMP status also affects patient survival. For example, patients with CIMP1 have a good prognosis because their disease consists mostly of MSI-H cancers. On the other hand, Barault *et al.* showed that CIMP status was an independent predictor of a poor prognosis in microsatellite stable colorectal cancer [27]. The unique characteristics of CIMP-positive tumors have also been reported in other malignancies. For example, Terada *et al.* reported that breast cancers with CIMP show a significantly higher rate of HER2 amplification [28], while gastric cancers with high levels of methylation rarely show mutations of *K-ras* or *p53* [29]. In general, methylation of multiple CpG islands is associated with a poor prognosis in patients with multiple malignancies – for example, acute leukemia with lung cancer and prostate cancer with esophageal cancer [30].

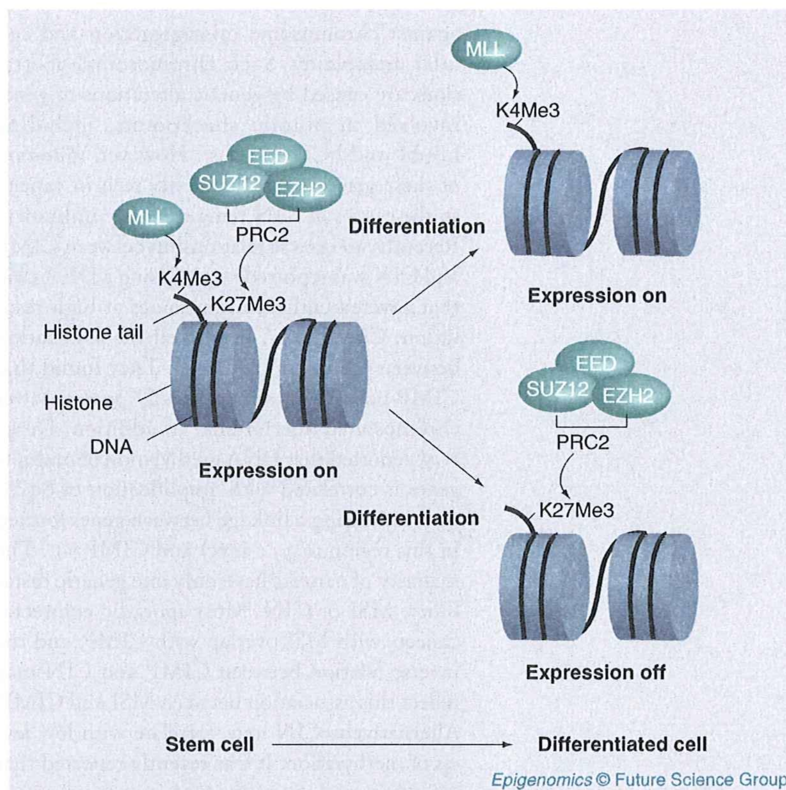


**Figure 2. The role of PRC2 during development.** In pluripotent embryonic stem cells, the promoter regions of some genes are modified to show methylation of histone H3 lysines 4 and 27. After differentiation, methylation of histone H3 lysine 4 is associated with gene expression, and methylation of histone H3 lysine 27 is associated with gene repression.
MLL: Mixed lineage leukemia.

There is a tight link between CIMP and *K-ras*/*BRAF* mutations, which makes it reasonable to speculate that mutations in the K-ras/BRAF pathway may induce aberrant methylation in cancer. In fact, Ras signaling has been shown to activate effectors mediating epigenetic silencing, including DNMT1, which plays a key role in cellular transformation [31,32]. This suggests that *K-ras*/*BRAF* activation may be involved in the CIMP phenotype through activation of the DNA methylation machinery. Alternatively, genes affected by CIMP may be involved in the cellular transformation associated with K-ras/BRAF activation. Activation of the K-ras/BRAF pathway induces senescence in cultured primary human cells. In addition, although *K-ras*/*BRAF* mutations are frequently seen in early lesions of the colon, these tumors undergo senescence. It has therefore been suggested that genes involved in the induction of senescence by K-ras/BRAF are altered during the progression of tumors [33]. For example, several genes involved in Ras-mediated senescence are inactivated by DNA methylation. Thus, colorectal cancers with CIMP may escape senescence by both activating oncogenic signaling (e.g., *BRAF* mutations) and inactivating regulators of senescence (e.g., p16 methylation).

The interaction between CIMP and APC mutations is not as well established as that between CIMP and the K-ras/BRAF pathway, though it is known that the presence of APC mutations is inversely correlated with the presence of *BRAF* mutations and CIMP [34]. Because CIMP tumors with *BRAF* mutations show large numbers of methylated CpG islands, one might expect WNT signaling to be defective in these tumors due to epigenetic inactivation of multiple WNT-regulating genes [35,36].

## Role of DNA methylation in chromosomal instability

Changes in genome integrity result in large chromosomal gains or losses, a phenomenon termed chromosomal instability (CIN). The integrity of chromosomes is maintained by mitotic checkpoints, which are an important cell cycle control mechanism that protects

against chromosome missegregation and cellular aneuploidy. Such chromosomal aberrations are caused by genetic alterations of genes involved in mitotic checkpoints, including hBub1 and hCDC4 [37,38]. However, mutation of these genes is not frequently seen in cancer, so the causes of CIN remain largely unknown. Recently, an inverse relationship between CIMP and CIN was reported [39,40]. Using a DNA chip that covered entire chromosomes at high resolution, Cheng *et al.* examined the association between CIN and CIMP [40]. They found that CIMP-positive tumors generally possess fewer chromosomal aberrations. In addition, Derks *et al.* reported that DNA methylation of multiple genes is correlated with amplification of 8q23-qter, indicating a linkage between genes located in this region (e.g., c-myc) and CIMP [41]. The majority of cancers have only one genetic instability, MSI or CIN. Most sporadic colorectal cancers with MSI overlap with CIMP, and the inverse relation between CIMP and CIN may reflect this association between MSI and CIMP. Alternatively, CIN may correlate with low levels of methylation. It was recently reported that levels of LINE1 methylation are significantly reduced in colorectal cancer without MSI [42], and that genome-wide demethylation is linked to chromosomal instability in colorectal cancer [43]. This is consistent with the findings that a global loss of methylated cytosines is one of the earliest epigenetic alterations in colorectal cancer [11], and that low levels of DNMT1 induce a loss of heterozygosity at the Apc locus in mice [44]. Further study will be necessary to clarify the relation between DNA methylation and chromosomal abnormalities in cancer.

## Involvement of pathogens in epigenetic alterations in gastric cancer

The causes of aberrant DNA methylation in cancer remain largely unknown. Cancers related to viral infections, including hepatocellular cancer [45], cervical cancer [46] and adult T-cell leukemia [47], all show methylation of multiple CpG islands. However, among the pathogen-associated cancers, the link between infection and epigenetic alterations is best characterized in gastric cancer [29,48–50]. In gastric cancer, mutations in

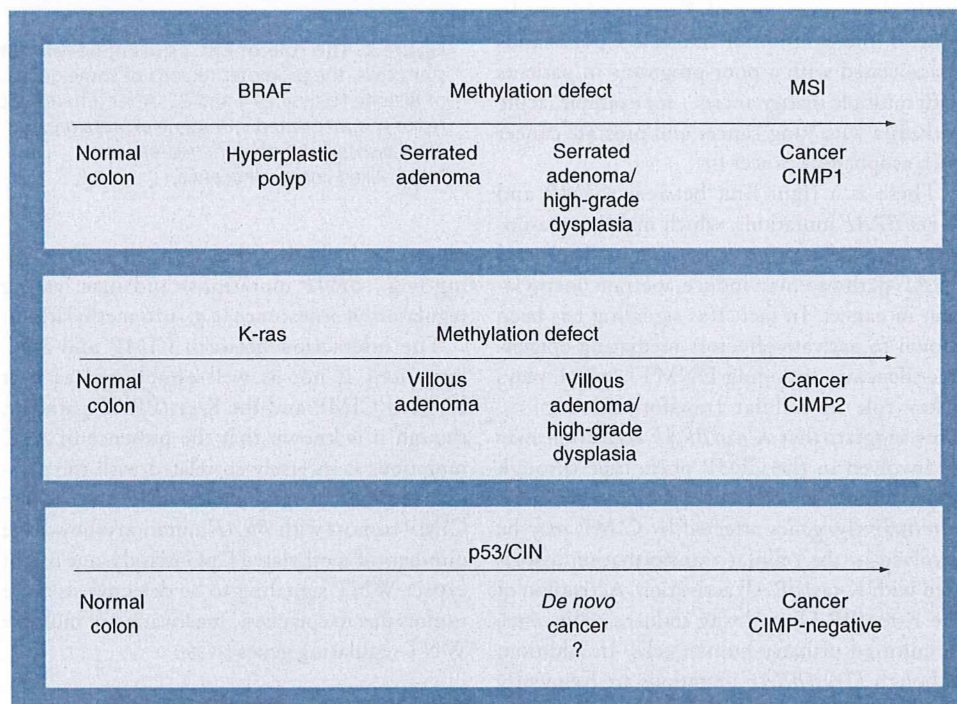


**Figure 3. Three pathways mediating colorectal tumorigenesis.** Based on the CIMP status, the development of colorectal cancer can proceed along three distinct pathways: CIMP1 tumors show a high degree of DNA methylation, microsatellite instability due to methylation of *hMLH1* and *BRAF* gene mutations; CIMP2 tumors show heterogeneous DNA methylation and frequent *K-ras* mutations; and CIMP-negative tumors show frequent *p53* mutations. These three types of tumors have distinct precursors: serrated adenoma for CIMP1, villous adenoma for CIMP2 and tubular adenoma for CIMP-negative.
CIMP: CpG island methylator phenotype; CIN: Chromosomal instability; MSI: Microsatellite instability.