

## The Epigenome as a Target for Cancer Chemoprevention

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**Epigenetic events, a key driving force in the development of cancer, are alterations in gene expression without changes in the DNA coding sequence that are heritable through cell division. Such changes occur throughout all stages of tumorigenesis, including the early phases, and are increasingly recognized as major mechanisms involved in silencing tumor suppressor genes. Epigenetic changes can be reversed by the use of small molecules and, thus, such changes are promising targets for cancer chemopreventive drug development. This review examines the basis for targeting the epigenome as a prevention strategy, focusing on understanding the epigenetic changes that occur before the development of frank malignancy, when chemopreventive intervention will have the maximal impact. [J Natl Cancer Inst 2003;95:1747-57]**

Two changes integral to epigenetic transcriptional control are DNA methylation and covalent modification of histone proteins (1,2). In cancer cells, genome-wide hypomethylation is accompanied by hypermethylation of short CpG-rich regions known as CpG islands. These islands, found in the promoter region of about half of genes, are generally unmethylated in normal cells. However, hypermethylation of CpG islands has been observed in nearly every type of human tumor, with unique patterns of individual gene methylation exhibited by each tumor type (3,4). Hypermethylation of promoter regions is associated with transcriptional silencing and is at least as common as mutation as a mechanism for inactivation of classic tumor suppressor genes in human cancers (1). Furthermore, a number of candidate tumor suppressor genes that are not commonly inactivated by mutation are transcriptionally silenced by this mechanism (1). Hypermethylation and silencing of genes involved in DNA repair (e.g., MGMT and hMLH1) can set the stage for tumor development by predisposing to mutations (5,6). A broad spectrum of other genes are also aberrantly methylated in cancers, including those associated with the cell cycle (e.g., retinoblastoma, p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, and p14<sup>ARF</sup>), signal transduction (e.g., RASSF1, LKB1/STK11, and APC), carcinogen metabolism (e.g., GSTP1), apoptosis (e.g., DAPK and CASP8), hormone response (e.g., ER, PGR, AR, and RAR $\beta$ ), angiogenesis (e.g., THBS1), and invasion and/or metastasis (e.g., TIMP3 and CDH1) (7,8).

The second major layer of epigenetic transcriptional control that has been widely studied is modification of histone proteins. These proteins serve as building blocks to package eukaryotic DNA into repeating nucleosomal units that are folded into higher order chromatin fibers. Histones undergo elaborate post-translational modifications on their amino-terminal tails, including acetylation, methylation, phosphorylation, and ubiquitination. Acetylation has been the most extensively studied and is

controlled by histone acetyltransferases and histone deacetylases. Acetylation is associated with nucleosome remodeling and transcriptional activation, whereas deacetylation is associated with transcriptional repression via chromatin condensation. In addition to their histone-modifying activity, histone deacetylases may also control gene expression by deacetylating transcription factors and contributing to cell cycle regulation (2,9).

Functional alterations of proteins in the histone acetyltransferase family (e.g., CREB-binding protein and p300) via mutation or the action of viral proteins are associated with certain cancers. Functional mutations and/or loss of heterozygosity in the CREB-binding protein gene are linked with precancerous Rubinstein-Taybi syndrome and hepatocellular carcinoma, and p300 is associated with glioblastoma and breast and colorectal cancers. Chromosomal translocations of both CREB-binding protein and p300 also occur in leukemias. Aberrant transcriptional repression mediated by histone deacetylases is associated with several hematologic malignancies, most notably, acute promyelocytic leukemia (2,10).

The two layers of epigenetic control, DNA methylation and histone acetylation, are integrally linked. Methylation is catalyzed by a family of DNA methyltransferases. DNA methyltransferases recruit histone deacetylases, leading to histone deacetylation and transcriptional repression. Methylated DNA is also recognized by a family of methylated DNA-binding proteins, which recruit histone deacetylases and ATP-dependent chromatin remodeling proteins, resulting in a tightly condensed chromatin structure and gene inactivation [for review, see (2)]. Additional links between the "histone code" and the "cytosine methylation code" are increasingly evident (1). A recent study (11) suggests that DNA methylation acts to lock in rather than initiate epigenetic silencing.

### EPIGENETIC CHANGES IN HIGH-RISK TISSUES: TARGETS FOR CHEMOPREVENTION

Epigenetic changes described in preinvasive lesions and/or high-risk tissues with the potential to serve as targets for chemoprevention are discussed below.

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**Table 1.** DNA methylation in the colon during aging and colorectal cancer development\*

Reference	Gene(s) methylated/CIMP status	Reference	Gene(s) methylated/CIMP status
(12)	ER gene methylated in aging normal mucosa and also methylated in 12/12 (100%) small and large sporadic adenomas and 26/26 (100%) sporadic cancers.	(16)	38/64 (59%) adenomas were CIMP-positive (average number of methylation events per adenoma was the same as for CIMP-positive cancers); 73% of large adenomas were CIMP-positive, and 23% of small adenomas were CIMP-positive.
(14)	N33 and MYOD methylated in aging normal mucosa and increased in adenomas and cancers.		KRAS mutation was more frequent in CIMP-positive than in CIMP-negative adenomas; p16 <sup>INK4a</sup> methylated only in CIMP-positive adenomas; hMLH1 was not methylated in adenomas.
(5)	hMLH1 partially methylated in normal-appearing mucosa of 15/34 (44%) colon cancer patients <60 years old and 20/24 (83%) patients ≥80 years old.	(109)	p14 <sup>ARF</sup> methylated in 0/32 (0%) normal-appearing mucosa, 13/41 (32%) adenomas, and 31/110 (28%) carcinomas.
	hMLH1 fully methylated in 18/33 (55%) patients with MSI-positive tumors and 18/90 (20%) patients with MSI-negative tumors.	(110)	COX2 methylated in 7/50 (14%) adenomas and 12/92 (13%) cancers; methylation was strongly associated with CIMP.
(106)	CDH13 methylated in 2/33 (6%) normal-appearing mucosa, 8/19 (42%) adenomas, and 17/35 (49%) cancers.	(87)	SFRP1 (11/17 [65%]), SEZ6L (2/13 [15%]), and KIAA0786 (4/8 [50%]) methylated in normal-appearing mucosa of patients whose tumors contained methylated alleles; methylation was likely a field effect.
(21)	HLTF methylated in 7/28 (25%) adenomas (all ≥1.5 cm in diameter and/or with villous histology) and 27/63 (43%) cancers.	(17)	Methylation of ≥1 loci in 52/108 (48%) adenomas and methylation of ≥2 loci in 19/76 (25%) adenomas.
(22)	Methylation of one or more (of six) genes in 21/61 (34%) ACF; more frequent methylation in dysplastic, sporadic ACF. Strong association of ACF methylation with KRAS mutation and chromosome 1p loss; methylation was the only molecular change in 10/61 (16%) ACF.		p16 <sup>INK4a</sup> methylated in 19/71 (27%) adenomas. Methylation status of different adenomas from same patient was not
(18)	MGMT methylated in 9/21 (43%) adenomas <1 cm in diameter, 23/44 (52%) adenomas ≥1 cm in diameter, and 71/179 (40%) carcinomas. In large adenomas or carcinomas, but not in small adenomas, MGMT methylation was associated with G → A mutations in KRAS.		Methylation of ≥1 loci in 17/23 (74%) tubulovillous or villous adenomas versus methylation in 35/85 (41%) tubular adenomas.
(19)	MGMT methylated in 126/314 (40%) colorectal lesions (249 carcinomas and 65 adenomas); methylation was associated with G → A transition mutations in p53.	(24)	Normal-appearing mucosa: methylation of ≥2 loci (CIMP-high) in 1/16 (6%) specimens, one locus (CIMP-low) in 2/16 (13%) specimens, and no loci in 13/16 (81%) specimens.
(20)	APC methylated in 0/28 (0%) normal-appearing mucosa, 9/48 (19%) adenomas (3/18 small and 6/30 large), and 20/108 (19%) cancers.		Sporadic HPs were CIMP-negative.
(107)	p16 <sup>INK4a</sup> methylated in 0/22 (0%) normal-appearing mucosa or normal mucosa, 1/6 (16%) adenomas (methylated in one large lesion but not in five small lesions), and 8/20 (40%) carcinomas.		HPs from patients with multiple (5–10) and/or large (>1 cm in diameter) HPs or hyperplastic polyposis (>20 polyps): 44/102 (43%) were CIMP-high, 14/102 (14%) were CIMP-low, and 44/102 (43%) were CIMP-negative.
(108)	p16 <sup>INK4a</sup> methylated in 0/9 (0%) normal mucosa, 2/8 (25%) normal-appearing mucosa, 7/13 (54%) adenomas, and 6/11 (55%) cancers; methylation was associated with KRAS mutation.		Methylation status was associated with different loci in the same HP and different HPs in the same patient. CIMP-high HPs were observed largely in patients with a predominance of HPs in the right colon and/or serrated adenomas and were associated with the absence of KRAS mutations.
(13)	p16 <sup>INK4a</sup> methylated in 3/15 (20%) adenomas; hMLH1 methylated in adenomas; 7/15 (47%) adenomas were CIMP-positive.		

\*ACF = aberrant crypt foci; CIMP = CpG island methylator phenotype; HP = hyperplastic polyp; MSI = microsatellite instability.

## Colorectum

Aberrant epigenetic regulation during tumorigenesis has been studied extensively in the colon (Table 1). Changes in DNA methylation appear to play two distinct roles during colorectal carcinogenesis. The first is a progressive, age-related methylation that silences a subset of genes in normal colorectal tissue (i.e., type A methylation). These age-related changes, first observed for the ER gene (12), have also been described for CSPG2, EGFR, IGF2, MYOD1, N33, PAX6, and RARβ2 genes. It has been hypothesized that type A methylation in the normal colon contributes to the increased risk of colorectal cancer associated with aging (13–15).

Apart from age-related methylation, a number of genes are uniquely methylated in cancers and/or preinvasive lesions. These “type C” genes include APC, CACNA1G, CALCA, H1C1, MGMT, TIMP3, and WT1 (13,16,17). Methylation of hMLH1 is reportedly both age-related and tumor-specific (5,13). The frequency of methylation observed in cancers is lower for

type C genes (10%–50%) than for type A genes (30%–100%). Type C methylation occurs in a subset of colorectal tumors that are defined as having the CpG island methylator phenotype. Colon cancers with the CpG island methylator phenotype include the majority of tumors with sporadic mismatch repair deficiency and KRAS mutations. Colon cancers that do not express the CpG island methylator phenotype develop along a more classic genetic instability pathway, with a high rate of p53 mutations and chromosomal changes. The CpG island methylator phenotype is observed in about 50% of colonic adenomas with synchronous cancers and in 25% of adenomas in the absence of cancer and is more common in adenomas with increased malignant potential. However, methylation is not associated with multiple adenomas from the same individual, suggesting that this epigenetic change is not a field defect in these lesions (13,16,17). Hypermethylation of the DNA repair gene MGMT also occurs frequently in adenomas and predisposes these tumors to KRAS (18) and p53 (19) mutations. The

**Table 2.** DNA methylation in patients with ulcerative colitis (UC)

Reference	Gene(s) methylated
(25)	Methylation of type A genes in normal mucosa, normal-appearing mucosa from UC patients, and high-grade dysplastic areas from UC patients, respectively: 7%, 20%, and 40% for the ER gene; 3%, 18%, and 44% for MYOD; 2%, 8%, and 9% for p16 <sup>INK4a</sup> ; and 31%, 35%, and 58% for CSPG2.
(26)	p14 <sup>ARF</sup> methylated in 3/5 (60%) non-neoplastic UC mucosa, 4/12 (33%) dysplasias, and 19/38 (50%) carcinomas.
(27)	p16 <sup>INK4a</sup> methylated in 13% of samples without dysplasia, 40% of samples indefinite for dysplasia, 14% of samples with only diploid cell populations, 70% of dysplasias, and 100% of carcinomas (total of 89 tissue samples obtained from three colectomy specimens).
(111)	CDH1 methylated in 13/14 (93%) colonoscopy specimens with dysplastic biopsies and in 1/17 (6%) specimens without dysplasia obtained from 26 UC patients (31 total colonoscopy specimens) but in 0/15 (0%) colonoscopy specimens from control subjects with irritable bowel syndrome.

classic colorectal gatekeeper APC gene can also be inactivated epigenetically in adenomas (20). Interestingly, the candidate tumor suppressor HMTF gene, a member of the SWI/SNF (mating-type switching/sucrose nonfermenting) family of chromatin remodeling proteins, is methylated in high-risk adenomas and cancers (21). Thus, the epigenetic machinery itself may be a target of epigenetic regulation.

Importantly, recent evidence also suggests that epigenetic changes occur at the preadenoma stages of colorectal cancer. Altered patterns of DNA methylation are observed in early preinvasive aberrant crypt foci. Methylation is more frequent in dysplastic aberrant crypt foci and in lesions associated with sporadic cancers than in familial cancers (22). Some hyperplastic polyps, now thought to represent preinvasive lesions developing along an alternative pathway from the classic adenoma-carcinoma sequence (23), may arise from epigenetic field defects (24).

Aberrant methylation patterns are also observed in patients with ulcerative colitis, a chronic inflammatory condition of the large intestine which predisposes to cancer (Table 2). Changes include altered methylation of type A genes in the normal-appearing mucosa and in high-grade dysplastic areas (25), as well as of type C genes, such as the tumor suppressors p14<sup>ARF</sup> and p16<sup>INK4a</sup> (26,27).

Genetically reducing expression levels of the predominant DNA methyltransferase Dnmt1 inhibits intestinal tumor development in mouse models of familial adenomatous polyposis (28) and hereditary nonpolyposis colon cancer (29); in the former, complete suppression of polyp formation was achieved. In both studies, Dnmt1 mutant mice exhibited a reduced frequency of CpG island hypermethylation in normal intestinal mucosa and tumors. It should be noted that, in addition to maintenance of DNA methylation, Dnmt1 can directly inhibit transcription (30), which may also contribute to the effects observed.

## Breast

Quantitative and gene-specific epigenetic changes are also observed early in the progression of breast cancer (Table 3). In a study of four genes, the putative tumor suppressor gene RASSF1A was the most frequently and heavily methylated

**Table 3.** DNA methylation during breast cancer development\*

Reference	Gene(s) methylated
(32)	14-3-3 $\sigma$ methylated in 0/5 (0%) hyperplasias without atypia, 3/8 (38%) atypical hyperplasias, 15/18 (83%) DCISs, and 24/25 (96%) carcinomas; also methylated in normal-appearing epithelium, stroma, and peripheral white blood cells; unmethylated in control epithelium.
(34)	CCND2 methylated in 8/18 (44%) DCISs and 8/17 (47%) carcinomas from 13 primary breast tumors that contained both invasive and noninvasive components; concordant methylation in DCIS and carcinoma; unmethylated in normal-appearing epithelium.
(31)	RASSF1A and 14-3-3 $\sigma$ methylated in epithelial hyperplasia, intraductal papillomas, and DCIS, but 14-3-3 $\sigma$ also methylated in stroma and lymphocytes. CCND2 only methylated in DCIS, and increased methylation was associated with increased histologic grade. p16 <sup>INK4a</sup> rarely methylated in DCIS.
(35)	ER methylated in 12/35 (34%) DCISs, 25/48 (52%) invasive ductal carcinomas, and 17/28 (61%) locally advanced or metastatic cancers. CDH1 methylated in 11/35 (31%) DCISs, 25/48 (52%) invasive ductal carcinomas, and 17/28 (61%) locally advanced or metastatic cancers. Methylation of both ER and CDH1 increased from 21% in DCIS to 50% in metastases.
(33)	At least one of three genes (CCND2, RAR $\beta$ , and TWIST) methylated in ductal lavage fluid from 5/45 (11%) healthy ducts and in fluid from ducts containing endoscopically visualized DCIS (2/7 [29%] specimens) or carcinomas (17/20 [85%] specimens). Two high-risk women with healthy mammograms whose ductal lavage fluid contained methylated markers and cytologically abnormal cells were subsequently diagnosed with cancer.
(112)	TRB1 methylated in 4/11 (36%) normal-appearing tissues and in 11/11 (100%) tumors but not in normal control tissues.

\*DCIS = ductal carcinoma *in situ*.

locus. Methylated alleles were present to a similar extent in intraductal papillomas and epithelial hyperplasias, as well as in ductal carcinoma *in situ*, but never in normal breast tissue (31). The gene for the negative cell cycle regulator 14-3-3 $\sigma$  was hypermethylated in intraductal papillomas, epithelial hyperplasias, and ductal carcinoma *in situ*; however, it was also hypermethylated in lymphocytes and stromal tissue (31,32). By contrast, aberrant methylation of the CCND2 gene was restricted to ductal carcinoma *in situ*, with increased methylation associated with higher histologic grade tumors; however, p16<sup>INK4a</sup> was only rarely methylated in ductal carcinoma *in situ* lesions. When intraductal and invasive tumor cells were compared, the methylation status of p16<sup>INK4a</sup>, 14-3-3 $\sigma$ , and RASSF1A genes was generally similar; however, variable quantitative changes in methylation of the CCND2 gene were detected (31). Others have confirmed methylation of the CCND2 gene in ductal carcinoma *in situ* (33,34) and in ductal fluid collected by routine operative breast endoscopy (33). The estrogen receptor  $\alpha$  and CDH1 genes are also methylated in about 30% of ductal carcinomas *in situ*, which increases to about 50% and 60% in invasive and metastatic lesions, respectively (35). A direct association between breast cancer tumor suppressor genes BRCA1 and BRCA2 and the epigenetic machinery has been found. BRCA1 interacts with histone-modifying enzymes (36,37) and components of the

chromatin-remodeling machinery (38) and can mediate large-scale chromatin decondensation (39). BRCA2 has intrinsic histone acetyltransferase activity (40).

Of particular interest is the epigenetic silencing of the RAR $\beta$  gene during breast cancer development. The antiproliferative and differentiative effects of retinoids, well-recognized chemopreventive agents, are mediated largely through interactions with members of the nuclear retinoic acid receptor family (41). Loss of RAR $\beta$  expression is an early event during breast carcinogenesis (42), which is mediated, at least in part, by epigenetic mechanisms (33). RAR $\beta$  is methylated in invasive cancers and ductal carcinoma *in situ* but is not methylated in normal breast tissue. Overall methylation of at least one of three genes (RAR $\beta$ , CCND2, or TWIST) was found in 48 of 50 invasive breast cancers and in eight of 14 ductal carcinomas *in situ* but was not found in healthy breast tissue. Methylation of RAR $\beta$  was also detected in ductal fluid collected by routine operative breast endoscopy from invasive ductal carcinoma *in situ* and atypical ductal hyperplastic lesions. Fluid from routine operative breast endoscopies contained methylated RAR $\beta$ , CCND2, or TWIST alleles in 17 of 20 invasive lesions, in two of seven ductal carcinomas *in situ*, and in two of six atypical ductal hyperplasias. Importantly, two high-risk women with healthy mammograms whose ductal lavage fluid contained methylated markers and cytologically abnormal cells were subsequently diagnosed with breast cancer (33).

## Prostate

The GSTP1 gene, which codes for the drug detoxification enzyme glutathione S-transferase  $\pi$ , is hypermethylated in the vast majority of prostate cancers and in a large number of preinvasive prostatic lesions (43–45) (Table 4); in one study (43), it was found in up to 70% of prostatic intraepithelial neoplasias. No mutations or deletions have been reported in the GSTP1 gene, suggesting that methylation is a major mechanism of gene inactivation. Loss of GSTP1 expression is closely associated with promoter hypermethylation in cancers; however, this tight association is lost in prostatic intraepithelial neoplasias (45). The discrepancy between gene expression and promoter hypermethylation may reflect higher levels of GSTP1 methylation in cancers than in preinvasive lesions (46). Methylation of other genes, including RAR $\beta$ 2, RAR $\beta$ 4, RASSF1A, CDH13,

**Table 4.** DNA methylation during prostatic cancer development\*

Reference	Gene(s) methylated
(43)	GSTP1 methylated in 0/54 (0%) normal-appearing tissues, 7/10 (70%) high-grade PINs, and 42/44 (95%) cancers.
(44)	GSTP1 methylated in 0/10 (0%) BPHs, in 4/6 (67%) PINs, and in 10/10 (100%) cancers.
(46)	GSTP1 quantitative methylation (median ratio MSP-amplified GSTP1/MYOD1) = 0 (range = 0–0.1) in BPH, 1.4 (range = 0–45.9) in PIN, and 250.8 (range = 53.5–697.5) in cancers.
(84)	GSTP1 methylated (after prostatic massage) in urine sediments of 1/45 (2%) BPHs, 2/7 (29%) PINs, 15/22 (68%) early cancers, and 14/18 (78%) locally advanced or systemic cancers.
(45)	GSTP1 methylated in 9/43 (21%) BPHs, 17/34 (50%) PINs, and 89/105 (85%) cancers.

\*BPH = benign prostatic hyperplasia; MSP = methylation-specific polymerase chain reaction; PIN = prostatic intraepithelial neoplasia.

APC, CDH1, and FHIT, has been detected in cancerous tissues (47), but the status of these epigenetic changes in preinvasive lesions is unknown.

## Esophagus

Hypermethylation of the p16<sup>INK4a</sup> tumor suppressor gene is a common event in both esophageal adenocarcinoma and in preinvasive Barrett's esophagus (Table 5). Methylation is detected in metaplasias and is similar in all grades of dysplasia, suggesting that this epigenetic alteration occurs very early in tumor progression (48–51). In a recent study (52) using bioinformatic algorithms to identify genetic and/or epigenetic lesions that provide a selective advantage (as compared with those that “hitchhike” but are neutral for tumor progression), hypermethylation of the p16<sup>INK4a</sup> promoter provided a clear and strong advantageous effect on cells early in the progression of Barrett's esophagus.

An in-depth study (53) of the methylation status of 20 genes in different stages of Barrett's esophagus and/or associated adenocarcinoma identified distinct “epigenetic fingerprints” in different histologic stages of the disease. Some genes were minimally informative because the frequency of hypermethylation was less than 5% (p14<sup>ARF</sup>, CDH1, p15<sup>INK4b</sup>, GSTP1, hMLH1, COX2, and THBS1), completely absent (CTNNB1, RB1, TGFBR2, and TYMS1), or ubiquitous (HIC1 and MTHFR), regardless of tissue type. Genes with an intermediate frequency of hypermethylation (in all tissue types combined) were more informative, with methylation ranging from 15% (p16<sup>INK4a</sup>) to 60% (MGMT) of samples. This group could be further subdivided into three classes, defined by the absence (p16<sup>INK4a</sup>, ER, and MYOD1) or presence (CALCA, MGMT, and TIMP3) of methylation in normal-appearing esophageal and stomach tissue or by the infrequent methylation of normal esophageal mucosa accompanied by methylation in all normal

**Table 5.** DNA methylation during esophageal cancer development

Reference	Gene(s) methylated
(48)	p16 <sup>INK4a</sup> methylated in 0/10 (0%) normal tissues, 4/12 (33%) Barrett's metaplasias, 3/11 (27%) low-grade dysplasias, 3/10 (30%) high-grade dysplasias, and 18/22 (82%) adenocarcinomas.
(49)	p16 <sup>INK4a</sup> methylated in 27/41 (66%) Barrett's metaplasias, 21/45 (47%) indefinite or low-grade dysplasias, and 17/21 (81%) high-grade dysplasias.
(50)	p16 <sup>INK4a</sup> methylated in 2/67 (3%) normal-appearing tissues from patients with Barrett's metaplasia, 3/5 (60%) tissues indefinite for dysplasia, 10/18 (56%) low-grade dysplasias, and 3/4 (75%) high-grade dysplasias.
	p16 <sup>INK4a</sup> methylated in 9/10 (90%) patients with dysplasia at some time during surveillance and 0/42 (0%) patients without dysplasia during surveillance.
(113)	APC methylated in 0/20 (0%) normal tissues, 17/43 (40%) Barrett's metaplasias, 48/52 (92%) adenocarcinomas, and 16/32 (50%) squamous cell carcinomas.
(52)	p16 <sup>INK4a</sup> methylation has strong advantageous effects on Barrett's cells early in progression.
(53)	Different stages of disease characterized by unique epigenetic fingerprints. Normal or metaplastic tissues from patients with associated dysplasia or cancer have higher incidence of methylation than tissues from patients without evidence of progression.

stomach samples (APC). Different stages of disease were characterized by unique epigenetic changes in each gene class. Aberrant methylation occurred at numerous loci in the same tissue, suggesting a generalized deregulation of methylation control, but a discrete group of tumors with the CpG island methylator phenotype was not identified. Importantly, normal and metaplastic tissues from patients with associated dysplasia or cancer had a higher incidence of hypermethylation than similar tissues from patients without further evidence of progression, suggesting that hypermethylation may identify esophageal lesions at risk for progression.

## Lung

p16<sup>INK4a</sup> methylation is detected in the earliest cytologic stages of lung cancer (Table 6) and increases during disease progression (54,55). In one study (54), methylation increased from 17% in basal cell hyperplasias to 24% in squamous cell metaplasias to 50% in carcinomas *in situ* to 61% in squamous cell cancers and was associated with loss of expression in both tumors and precursor lesions. p16<sup>INK4a</sup> is also methylated in nonmalignant bronchial epithelium from current and former smokers and in sputum from high-risk individuals and patients with lung cancer (6,54,56–58) but not in the bronchial epithelium of never smokers (56). Furthermore, p16<sup>INK4a</sup> methylation has been detected in sputum up to 3 years before the diagnosis of cancer (58) and in hyperplasias, adenomas, and adenocarcinomas from rats treated with tobacco-specific carcinogens (54).

The strong association between p16<sup>INK4a</sup> methylation status in bronchial epithelium and corresponding primary tumors (56) and the high frequency of promoter methylation (up to 80% in cancers) [for review, *see* (59)] underscores the importance of this event for lung cancer development. Although p16<sup>INK4a</sup> methylation occurs early in lung tumorigenesis, the available data suggest that it is not predictive for cancer development in this tissue but is likely permissive for acquisition of additional genetic and/or epigenetic changes (6,55,56). Altered methylation of additional genes, including DAPK, MGMT, and FHIT, has also been implicated in the early stages of lung cancer progression (6,56,60).

## Cancers Associated With Inflammation and/or Infection

In addition to being observed in ulcerative colitis, aberrant methylation is seen in other chronic inflammatory conditions which predispose to cancer. Methylation of the p16<sup>INK4a</sup> promoter is found in chronic hepatitis and cirrhosis associated with hepatitis B and hepatitis C viral infections, which are risk factors for liver cancer. Importantly, analysis of serial samples from patients with methylation-positive hepatocellular carcinoma detected the loss of p16<sup>INK4a</sup> gene methylation and protein expression in 18 of 20 patients at the stage of chronic hepatitis that precedes clinically detectable carcinoma (61).

Changes in methylation of host genes occur in infection-related cancers in addition to hepatocellular carcinoma. For example, in malignant mesothelioma, methylation of RASSF1A is linked with simian virus 40 infection (62), and in gastric cancer, methylation of multiple host genes is associated with chronic Epstein–Barr virus infection (63).

**Table 6.** DNA methylation during lung cancer development\*

Reference	Gene(s) methylated
(54)	p16 <sup>INK4a</sup> methylated in 2/12 (17%) basal cell hyperplasias, 4/17 (24%) squamous metaplasias, 3/6 (50%) CISs, and 11/18 (61%) SCCs. p16 <sup>INK4a</sup> methylated in sputum samples from 3/7 (43%) cancer patients and 5/26 (19%) cancer-free smokers.
(6)	p16 <sup>INK4a</sup> methylated in 11/11 (100%) sputum samples collected 5–35 months before cancer diagnosis; 90% concordance between p16 <sup>INK4a</sup> methylation in SCCs and in sputum. MGMT methylated in 7/11 (64%) sputum samples collected 5–35 months before cancer diagnosis; 78% concordance between MGMT methylation in SCCs and in sputum. In sputum from cancer-free, tobacco- and/or radon-exposed individuals, p16 <sup>INK4a</sup> was methylated in 18/123 (15%) individuals, and MGMT was methylated in 31/123 (25%). Of three lung cancers subsequently diagnosed, two were SCCs and one was adenocarcinoma. MGMT was methylated in sputum samples collected 1–3 years before cancer diagnosis in both patients with SCC; neither gene was methylated in sputum collected 2 years before diagnosis in the patient with adenocarcinoma.
(55)	p16 <sup>INK4a</sup> methylated in 1/22 (5%) normal or inflamed bronchial biopsies, 5/20 (25%) metaplasias, 5/24 (21%) mild or moderate dysplasias, and 2/4 (50%) severe dysplasias or CISs. No significant difference in p16 <sup>INK4a</sup> methylation between 3/6 (50%) progressing and 5/23 (22%) stable lesions.
(57)	p16 <sup>INK4a</sup> methylated in 17/100 (17%) bronchial brushes from cancer-free former smokers, DAPK methylated in 17/100 (17%), and GSTP1 methylated in 6/100 (6%). At least one of three genes tested was methylated in 32% of the samples, and two genes were methylated in 8% of the samples. p16 <sup>INK4a</sup> methylation was higher in former smokers with a prior history of cancer than in those without a prior history, and DAPK methylation was more frequent in older patients. No association was observed between methylation of any gene and smoking characteristics.
(56)	p16 <sup>INK4a</sup> and DAPK, respectively, methylated in nonmalignant cultured bronchial epithelium of 23/52 (44%) and 5/52 (10%) current or former smokers with cancer and in 18/41 (44%) and 4/41 (10%) current or former smokers without cancer. Methylation was as frequent in current smokers as in former smokers. No methylation was observed in epithelium of never smokers. Concordant methylation of p16 <sup>INK4a</sup> in the tumor and at least one bronchial epithelial site from lung lobes not containing the primary tumor was observed in 17/18 (94%) tumors. In sputum from cancer-free control subjects, p16 <sup>INK4a</sup> methylated in 23/66 (35%) subjects, DAPK methylated in 16/66 (24%) subjects (8/16 with p16 <sup>INK4a</sup> methylation), and MGMT methylated in 9/66 (14%) subjects. Abnormal sputum cytology or smoking status (current versus former) was not associated with methylation profile in sputum.
(60)	FHIT methylated in 6/35 (17%) bronchial brushes from cancer-free smokers (3/6 with mild dysplasia and 3/6 with moderate dysplasia) and in 40/107 (37%) with NSCLC.
(58)	p16 <sup>INK4a</sup> methylated in 4/25 (16%) sputum samples, 3/25 (12%) BALs, and 2/25 (8%) bronchial brushes from cancer-free smokers (total 7/25 [28%] patients); p16 <sup>INK4a</sup> methylated in 18/51 (35%) sputum samples, 11/51 (22%) BALs, and 8/51 (16%) bronchial brushes from cancer patients (total 26/51 [51%] patients). Three smokers with p16 <sup>INK4a</sup> methylation later developed lung cancer.

\*BAL = bronchoalveolar lavage; CIS = carcinoma *in situ*; NSCLC = non-small-cell lung cancer; SCC = squamous cell carcinoma.

## CANCER PREVENTION BY TARGETING THE EPIGENOME

Unlike tumor suppressor genes inactivated by genetic alterations, genes silenced by epigenetic mechanisms are intact and

responsive to reactivation by small molecules. Many diverse genes hypermethylated in cancers can be reactivated with DNA methyltransferase inhibitors (64). These agents, all analogs of the nucleoside deoxycytidine (Fig. 1, A), inhibit the growth of cancer cells *in vitro* and in animal models (64). Four demethylating agents have been tested clinically: 5-azacytidine, 5-aza-2'-deoxycytidine (decitabine), 1- $\beta$ -D-arabinofuranosyl-5-azacytosine (fazarabine), and dihydro-5-azacytidine. The first two show some efficacy in treating leukemia but show little activity against solid malignancies; trials of the latter two were stopped because of lack of efficacy (65). 5-Azacytidine and 5-aza-2'-deoxycytidine are unstable in aqueous solution, necessitating fresh preparation and administration via injection (66); moreover, their therapeutic use is limited by toxicity (64).

The orally available nucleoside analog zebularine, originally developed as a cytidine deaminase inhibitor, is also a potent inhibitor of DNA methylation (67). Zebularine suppresses growth of human bladder carcinoma xenografts in nude mice in association with reactivation of silenced p16<sup>INK4a</sup> and may be less toxic than the aforementioned nucleoside analogs (66). Non-

nucleoside drugs such as the antiarrhythmic procainamide also induce reexpression of methylation-silenced genes (68).

A limited number of preclinical studies have examined the ability of DNA methyltransferase inhibitors to prevent cancer. 5-Aza-2'-deoxycytidine markedly reduced tumor development in Apc<sup>Min/+</sup> mice, diminishing intestinal tumor formation by 82%. In Apc-deficient mice also heterozygous for Dnmt1, inhibition of tumor formation with 5-aza-2'-deoxycytidine was greater than 98%. Early administration is essential for chemopreventive activity; inhibition was lost when treatment was initiated at 50 days rather than 7 days of age (69). In other studies, 5-aza-2'-deoxycytidine diminished the formation of aberrant crypt foci in the colons of selenium-deficient rats treated with carcinogen (70) and prevented lung tumor formation in mice treated with a tobacco-specific carcinogen (71). 5-Aza-cytidine also reversed the immortal phenotype of a subset of cultured oral dysplastic cells together with inhibition of telomerase activity and reexpression of silenced RAR $\beta$  and p16<sup>INK4a</sup> (72).

The ability of histone deacetylase inhibitors (Fig. 1, B) to modify the epigenome is also being explored. These drugs

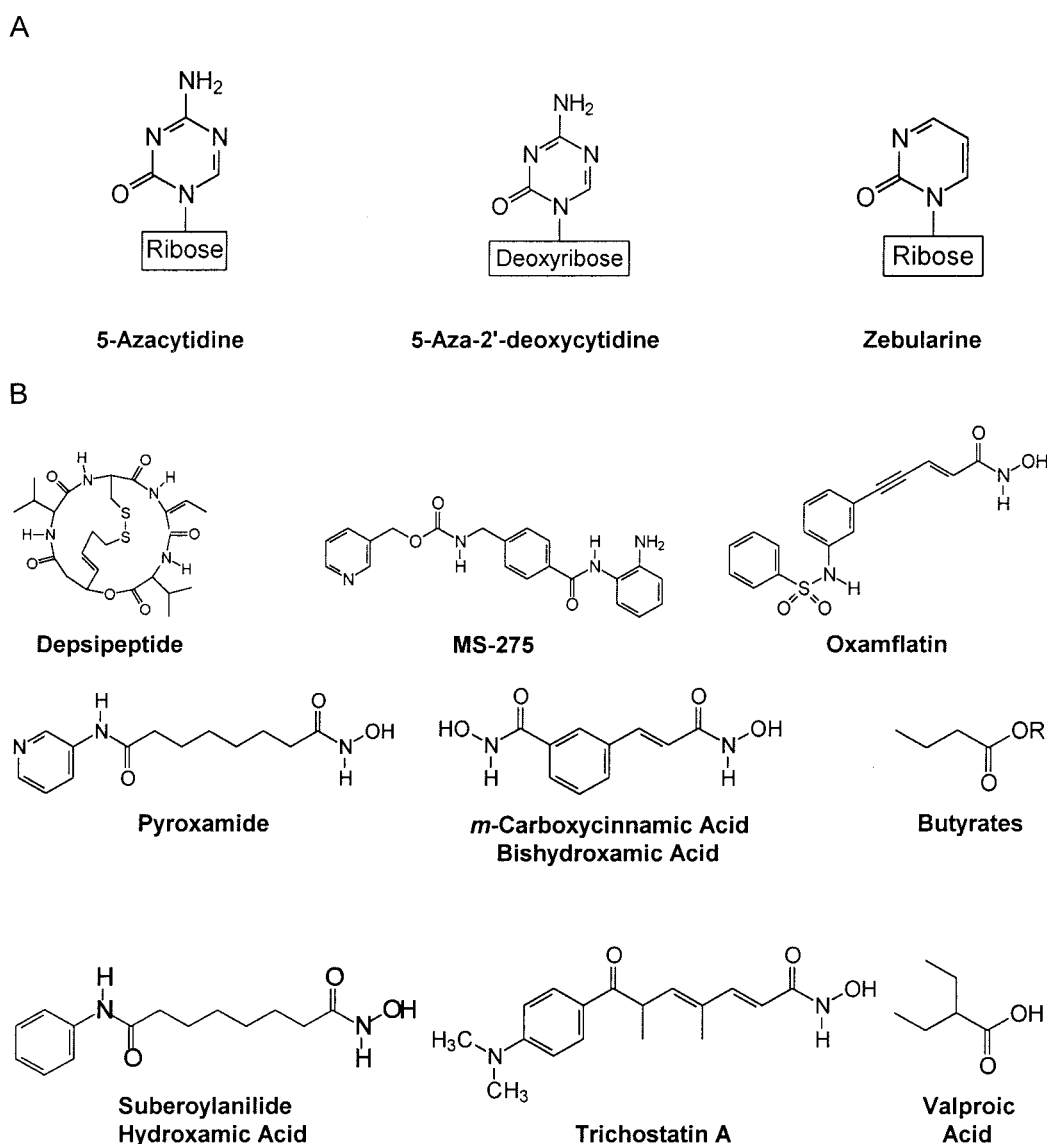


Fig. 1. A) DNA methyltransferase inhibitors. B) Representative histone deacetylase inhibitors.

induce cell-cycle arrest, apoptosis, and/or differentiation in transformed cells *in vitro* and suppress the growth of a wide variety of solid tumor xenografts with minimal toxicity. Several structural classes of histone deacetylase inhibitors have been identified, including short-chain fatty acids (butyric and valproic acids), hydroxamic acids (suberoylanilide hydroxamic acid, *m*-carboxycinnamic acid bishydroxamide, trichostatin A, oxamflatin, and pyroxamide), tetrapeptides (depsipeptide), and benzamides (MS-275, which stands for *N*-(2-aminophenyl)-4-[*N*-(pyridin-3-yl-methoxycarbonyl)aminomethyl]benzamide and CI-994, which stands for *N*-acetyldinaline). Phenylbutyrate, valproic acid, suberoylanilide hydroxamic acid, pyroxamide, depsipeptide, MS-275, and CI-994 are in clinical trials, and development of new histone deacetylase inhibitors is an active area of research (2,9,73). Interestingly, only 2%–10% of genes are expressed after exposure to histone deacetylase inhibitors. Activated genes are largely associated with regulation of cell growth and survival, providing a rationale for the antitumor actions of the histone deacetylase inhibitors; however, the mechanism for selectivity of gene activation remains unknown (2,74).

Several studies (75,76) have also examined the chemopreventive activity of histone deacetylase inhibitors. Suberoylanilide hydroxamic acid decreased the incidence and multiplicity of *N*-methyl-*N*-nitrosourea-induced rat mammary tumors; unlike 5-aza-2'-deoxycytidine, inhibitory effects were manifest at stages after initiation. Suberoylanilide hydroxamic acid also inhibited hyperplastic nodule formation in response to 7,12-dimethylbenz[*a*]anthracene treatment in mouse mammary gland organ culture (Mehta R, et al.: unpublished results) and the multiplicity of carcinogen-induced lung tumors in mice (77). However, the agent did not inhibit formation of carcinogen-induced aberrant crypt foci in the rat colon when fed at 300 mg/kg diet (Reddy B, et al.: unpublished results). While the histone deacetylase inhibitor phenylbutyrate diminished development of aberrant crypt foci in rat colon (78), under the conditions tested, it did not inhibit the formation of colon tumors (Reddy B, et al.: unpublished results). Changes in epigenetic regulation may also contribute to the efficacy of a number of well-recognized chemopreventive agents, including  $\alpha$ -difluoromethylornithine (79) and organosulfur (80) and selenium (81) compounds.

## CONCLUSIONS

It is clear that epigenetic changes are some of the earliest events observed during cancer development, making them excellent targets for chemoprevention. Populations that may benefit from this strategy include individuals who harbor genetic and/or acquired inflammatory or preinvasive lesions with aberrantly silenced DNA or who harbor infectious agents that alter the epigenome. A generalized increased cancer risk may also be associated with age-related changes in methylation patterns. In the latter, it will be important to determine specifically which age-related methylated genes predispose to cancer. The unique epigenetic fingerprint observed in patients with preinvasive esophageal lesions that progressed to more advanced lesions described above (53) underscores the potential use of epigenetic markers in risk assessment and early detection, and perhaps as surrogate end points in chemoprevention trials (82). The ability to detect aberrantly methylated DNA noninvasively (6,33,83,84) and the high degree of specificity of detection methods (85)

make this approach particularly attractive. Moreover, a new microarray-based assay combining gene expression and epigenetic regulation offers the potential to identify the entire spectrum of genes silenced by epigenetic mechanisms during cancer development (86).

However, the observation that precancerous tissues display global DNA hypomethylation [e.g., (87,88)] suggests that a cautious approach must be undertaken in developing epigenetic drugs as cancer preventives. DNA hypomethylation has been associated with chromosomal instability, reactivation of transposable elements (such as retroviral elements), loss of imprinting, and activation of protooncogenes (89). Of concern are several recent reports of increased tumorigenicity and chromosomal instability in mice carrying *Dnmt1* hypomorphic alleles (20,90,91). Mice bearing hypomorphic *Dnmt1* mutations and lacking the *Mlh1* mismatch repair gene, although protected against intestinal tumors, are at increased risk for lymphoma (29). The relevance of these studies to humans is unclear. Unlike mouse cells, human cancer cells lacking DNMT1 exhibit appreciable DNA methylation (92); indeed, DNMT1 and DNMT3b are both needed to maintain DNA methylation and gene silencing in human cancer cells (93). The rare recessive ICF (immunodeficiency, centromeric region instability, facial abnormalities) syndrome is caused by a mutation in the catalytic domain of the DNMT3b gene (94). Despite displaying increased chromosomal breakage, it is notable that patients with ICF syndrome do not appear to be at increased risk for cancer (95).

Given that preventive intervention with epigenetic drugs will likely require long-term administration to large populations at relatively low absolute risk for the development of cancer (96), establishment of acceptable chronic safety is essential. Combining demethylating agents and histone deacetylase inhibitors may provide a means to reduce the side effects associated with the former drugs. Synergy between 5-aza-2'-deoxycytidine and trichostatin A has been achieved in reactivating methylation-silenced tumor suppressor genes (97). Other promising strategies include the use of "synthetic lethality" (98) and the combined use of epigenetic drugs with agents that target reactivated genes—in effect using epigenetic drugs as cellular sensitizers (99). Restoration of response to retinoid signaling using this approach has been observed in acute myeloid leukemia (100) and in colon (101) and breast (102–104) cancers. The latter finding is particularly appealing, given that methylation-induced silencing of *RAR $\beta$*  is an early event during breast carcinogenesis (33). This approach could be used to reactivate other genes silenced in precancerous tissues that are targets for established chemopreventive agents. For example, the *ER* gene, which is silenced in a subset of early breast lesions (35), might be targeted by epigenetic drugs in combination with selective estrogen receptor modulators (105).

Permutations of these approaches and continued advancement in understanding the mechanisms involved in epigenetic regulation and how they interact with genetic changes during tumor progression will facilitate development of newer, more efficacious, and safer chemopreventive agents. The observation that epigenetic changes occur across a broad range of tissues during the early phases of cancer development (Tables 1–7) makes targeting the epigenome a promising and widely applicable preventive strategy.

**Table 7.** DNA methylation during cancer development: representative changes at miscellaneous target sites\*

Reference	Target site	Gene(s) methylated
(114)	Vulva	14-3-3 $\sigma$ methylated in 0/4 (0%) VIN-I, 2/6 (33%) VIN-II, 13/22 (59%) VIN-III, and 19/36 (53%) SCCs. p16 <sup>INK4a</sup> methylated in 0/4 (0%) VIN-I, 2/6 (33%) VIN-II, 9/22 (41%) VIN-III, and 13/36 (36%) SCCs.
(115)	Endometrium	hMLH1 methylated in 8/116 (7%) endometrial hyperplasias (primarily in atypical lesions with coexisting carcinoma) and 11/27 (41%) carcinomas; methylation was highly associated with MSI in cancers.
(116)	Cervix	Methylation of p16 <sup>INK4a</sup> , RAR $\beta$ , FHIT, GSTP1, MGMT, and hMLH1 was examined. All genes were unmethylated in control tissues; methylation of at least one gene was observed in 11/37 (30%) nondysplastic or low-grade CINs, 12/17 (71%) high-grade CINs, and 14/19 (74%) invasive cancers. Methylation of RAR $\beta$ and GSTP1 is an early event, methylation of p16 <sup>INK4a</sup> and MGMT is an intermediate event, and methylation of FHIT is a late, tumor-associated event.
(117)	Oral cavity	14-3-3 $\sigma$ unmethylated in normal-appearing epithelium and methylated in 3/6 (50%) dysplasias and 32/92 (35%) SCCs; p16 <sup>INK4a</sup> methylated in 2/6 (33%) dysplasias (one with 14-3-3 $\sigma$ methylation) and 38/92 (41%) SCCs.
(82)	Upper aerodigestive tract (larynx and oral cavity)	p16 <sup>INK4a</sup> methylated in 1/3 (33%) mild, 11/14 (79%) moderate, and 9/11 (82%) severe dysplasias. Methylation was reversed in 8/21 (38%) patients after treatment with 13- <i>cis</i> -retinoic acid, interferon $\alpha$ , and $\alpha$ -tocopherol for 12 months.
(118)	Bladder	DAPK methylated in 14/16 (88%) superficial bladder cancers recurring within 15 months, but 28/39 (72%) bladder cancers without methylation did not recur for 24 months. DAPK methylated in cancerous but not noncancerous regions.
(119)	Stomach	Methylation status was examined in chronic gastritis (n = 69), intestinal metaplasia (n = 49), adenomas (n = 61), and carcinomas (n = 64). DAPK methylation was similar in all stages. hMLH1 and p16 <sup>INK4a</sup> were not methylated in chronic gastritis and methylated in cancers more frequently than intestinal metaplasias or adenomas. p16 <sup>INK4a</sup> methylation higher in adenomas than in intestinal metaplasias. THBS1 and TIMP3 methylation markedly increased from chronic gastritis to intestinal metaplasia and from adenomas to carcinomas. hMLH1, THBS1, and TIMP3 methylation was similar in intestinal metaplasia and adenomas. Of five genes tested, the average number of methylated genes was 0.6, 1.1, 1.1, and 2.0 per sample in chronic gastritis, intestinal metaplasia, adenomas, and carcinomas, respectively. Marked increase in methylated genes was observed from nonmetaplastic mucosa to intestinal metaplasia and from preinvasive lesions to carcinomas.

\*CIN = cervical intraepithelial neoplasia; MSI = microsatellite instability; SCC = squamous cell carcinoma; VIN = vulval intraepithelial neoplasia.

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## NOTE

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