

The Biology Behind**The Potential Prognostic, Predictive, and Therapeutic Values of DNA Methylation in Cancer<sup>1</sup>**

**Commentary re: J. Kwong *et al.*, Promoter Hypermethylation of Multiple Genes in Nasopharyngeal Carcinoma. *Clin. Cancer Res.*, 8: 131–137, 2002, and H-Z. Zou *et al.*, Detection of Aberrant p16 Methylation in the Serum of Colorectal Cancer Patients. *Clin. Cancer Res.*, 8: 188–191, 2002.**

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In this issue of *Clinical Cancer Research*, Kwong *et al.* (1) studied DNA methylation in eight cancer-related genes in nasopharyngeal carcinoma. A second paper by Zou *et al.* (2) approaches the question of whether the presence of *p16* promoter methylation in the sera of patients with colorectal cancer can be a useful marker for early detection and prognosis. Both of these studies reflect the high incidence and underscore the potential clinical utility of altered DNA methylation patterns in cancer.

Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasia (3, 4). Cytosines are methylated in the human genome mostly when located 5' to a guanosine. These CpG nucleotides have been severely depleted in the vertebrate genome to ~20% of the predicted frequency, and most CpG dinucleotides (>70%) are methylated. However, in small stretches of DNA termed CpG islands, which are ~500 bp to 2000 bp in length (5), the CpG dinucleotide occurs at near the expected frequency, and these areas are frequently located in and around the transcription start sites of approximately half of the human genes. It has been increasingly recognized over the past 4–5 years that the CpG islands of a large number of genes, which are unmethylated in normal tissues, are methylated to varying degrees in multiple

types of human cancer (3, 6, 7). The postsynthetic covalent addition of a methyl group to cytosine is mediated by the three known active DNMTs (DNMT1, DNMT-3a, and DNMT-3b; Ref. 8).<sup>4</sup> These epigenetic “markers” on DNA can be copied after DNA synthesis, resulting in heritable changes in chromatin structure. The reciprocal relationship between the density of methylated cytosine residues and the transcriptional activity of a gene has been widely documented. However, it should be emphasized that this inverse correlation has been demonstrated conclusively only for methylation in the promoter regions and not in the transcribed parts of a gene (6).

Several tumor suppressor genes contain CpG islands in their promoters, and many of them show evidence of methylation silencing. After these CpG islands are methylated, and after changes associated with histone deacetylation have occurred, the relevant genes become silent. The methylation of cytosine nucleotides in human cancer cells can participate in the inactivation of the apoptosis pathway at several points, either upstream (at *p14<sup>ARF</sup>* or *DAP-kinase*; Refs. 9, 10) or downstream (*caspases*; Refs. 11) of the p53 or p53-independent (*RAR-β2*) pathways (12). Other genes frequently methylated in cancer are key players in cell cycle control (*p15* and *p16*) or are involved in DNA repair (*MGMT*, *hMLH1*, and *GSTP1*) and play important roles in protecting cells from carcinogenic agents. Quite a number of these genes are part of pathways, which are switched on by biological response modifiers (BRMs such as IFNs, retinoids, tumor necrosis factor, and so forth), and, thus, lead to cell death.

Methylation changes are sometimes associated with aging in the normal epithelium and have been described for instance for the *estrogen receptor*, *p16 exon 1* and *MYOD*. Age-related methylation clearly precedes neoplasia and is more prevalent in normal-appearing epithelium from cancer patients compared with normal epithelium from controls (13). Conflicting results are reported about the presence of MLH1 methylation in normal tissue (13, 14). These discrepancies most likely relate to differences in the population that were studied (normal tissue of cancer patients or noncancer patients) to the methods that have been used and to the regions where methylation has been studied. Thus, before DNA methylation can broadly be used for early detection, it will be necessary to show which DNA methylation patterns in normal-appearing tissues are associated with

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<sup>4</sup> The abbreviations used are: DNMT, DNA cytosine methyltransferase; RAR, retinoic acid receptor; BRM, biological response modifier.

carcinogenesis and which DNA methylation patterns are associated with age but do not predispose to cancer in general.

**DNA Methylation Analysis.** It has been notoriously difficult to accurately measure the level of methylation at given CpG sites within target areas of genes. Previously, methylation analysis relied on the use of methylation-sensitive restriction enzymes, which limited the number of CpG dinucleotides that could be assessed and was difficult in formalin-fixed material. This situation was changed by the invention of techniques for the conversion of unmethylated but not methylated cytosines to uracil by bisulfite treatment (15). There are now many new procedures based on bisulfite conversion that have significantly opened up this field (16). These include the sequencing of individual molecules of DNA so that the problem of allele specific methylation can be more adequately addressed (17). There are also very sensitive assays for determining methylation in given areas based on primers specific for converted DNA (methylation-specific PCR; Ref. 18) and techniques that allow a quantitative assessment at the level of methylation at individual CpG dinucleotides. These include (methylation-sensitive single nucleotide primer extension; Ref. 19) and a technique called combined bisulfite restriction analysis (20). However, all of the above methods are fairly labor-intensive.

Recently a more advanced technique called MethyLight that combines the advantages of quantitative accuracy of COBRA with the sensitivity of methylation-specific PCR has been developed (21). This is the only established technique that is compatible with automation and does not require gel electrophoresis. MethyLight relies on fluorescence-based real-time PCR ("TaqMan" technology) for the quantitative determination of the prevalence of hypermethylated alleles (22).

#### **DNA Methylation as a Means to Characterize a Tumor.**

There is an increasing necessity to better characterize a tumor of patients to provide prognostic as well as predictive information for better patient care. For instance, information on the status of steroid receptors in breast cancer has become an absolute requirement for the choice of the appropriate adjuvant therapy. Beside studying protein expression using radioligand-binding assays or immunology-based techniques, microarray-based RNA expression techniques makes it possible to study expression of thousands of genes within one tumor. Despite very promising results (23) there are potential pitfalls to the use of expression analysis in clinical routine including the need for preservation of mRNA species from the tissue of interest and the issue of tissue heterogeneity.

Tumor-specific DNA alterations might be a more promising strategy to better characterize a tumor, because DNA changes are stable, amplifiable, and very frequently, methylation changes are a surrogate for altered expression of the gene product.

Although Kwong *et al.* (1) were not able to identify a significant correlation between methylation status of the genes they examined and clinical outcome (this might also be attributable to the low number of patients and the relative short follow-up time), there is increasing evidence that the methylation status of certain genes in different tumor entities might provide independent prognostic information (24–27).

Beside studying methylation in the tumor, Zou *et al.* (2) provide evidence that the presence of *p16* methylation in serum

samples of patients with colorectal cancer correlates with later Dukes' stage. Although the authors did not provide outcome data, the presence of methylated DNA in plasma of esophageal patients was shown recently to be associated with decreased survival (28).

It is intriguing to speculate that the depth of invasion and subsequent increased likelihood of tumor cells accessing blood vessels is reflected in the presence of tumor-specific DNA in serum, blood, and bone marrow (Fig. 1). Currently there is an ongoing debate about the value of detecting tumor cells in bone marrow (29). It remains to be seen whether DNA methylation analysis in serum or plasma, because of the advantages of higher specificity, probably sensitivity, and less stress for the patient, might be a better tool to gain prognostic as well as predictive information.

#### **Early Detection of Aberrant DNA Methylation Patterns.**

Cytological screening for cervical cancer and its precursors has unequivocally shown how efficacious and cost-effective early detection can be. The effectiveness of this screening approach is mostly attributable to the fact that the "organ of interest" is easily accessible and that changes can easily be identified by a very well-established marker like cytology. This kind of analysis is not always applicable in other organ sites. A DNA-based approach might be promising, because DNA extracted from plasma, serum, or other body fluids of a patient could be easily amplified by PCR technology and is, therefore, potentially more sensitive. RNA is not as useful as a detection marker because of its inherent instability.

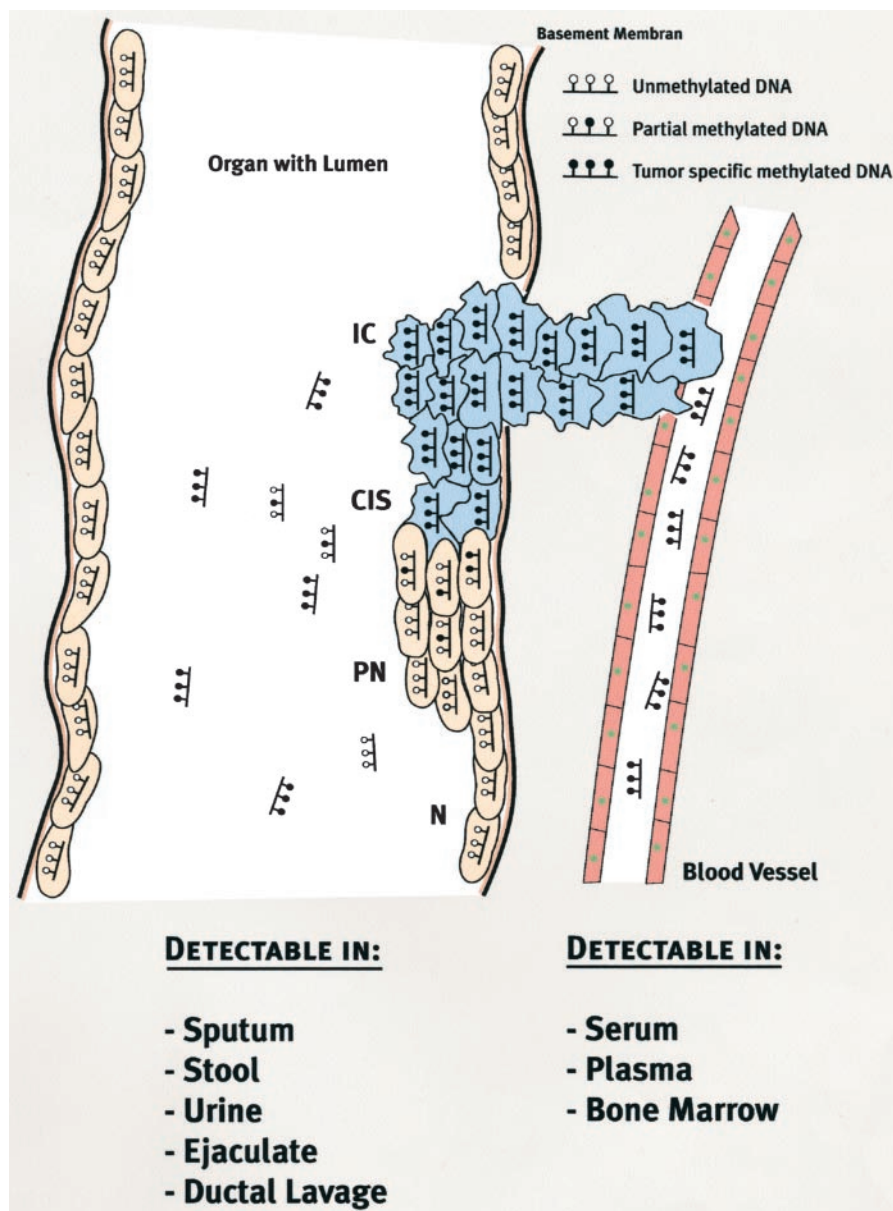
Detection of promoter CpG island hypermethylation offers several advantages compared with other DNA alterations in cancer. Abnormal DNA methylation represents a chemically and biologically stable tumor-specific marker that can be readily detected, independent of the level of gene expression. Once a tumor cell has acquired methylation of a specific gene, then this specific methylation pattern will generally not be lost and, indeed, is often enhanced during disease progression (30). Compared with point mutations, which often occur at different sites within a given gene in individual tumors, DNA methylation occurs mainly at specific CpG islands in the promoter regions of tumor suppressor genes. Finally, as compared with other frequent chromosome changes in cancer, such as allelic loss, CpG island hypermethylation constitutes a positively detectable signal, as opposed to a loss of signal, such as in loss of heterozygosity.

Methylated DNA can be detected with a very high degree of specificity, even in the presence of a vast excess of unmethylated DNA. MethyLight technology, for instance, can detect a single hypermethylated allele against a background of 10,000 unmethylated alleles (21).

There are several possible noninvasive ways to obtain DNA from tumors (Fig. 1). The most efficient method to detect DNA alterations for several malignancies is by examining DNA directly from the site where the cancer occurs. For example, breast cancer detection by ductal lavages (31), colorectal cancer by stool (32), lung cancer by sputum (33), and prostate cancer by urine or ejaculate (34).

Abnormalities have also been detected in tumor-derived DNA found in the serum of some cancer patients (35). Several studies have documented the presence of free DNA derived

*Fig. 1* Simplified illustration of different sources to detect tumor-specific methylated DNA. Normal epithelial cells (*N*) have unmethylated CpG islands, whereas preneoplastic cells (*PN*) can develop methylation of certain CpG islands. These methylation changes can be enhanced toward genesis of carcinoma *in situ* (*CIS*) and invasive cancer (*IC*). Depending on the site and stage of the tumor or the noninvasive lesion, methylated DNA can be studied either in the blood or in samples drained either physiologically or artificially to the outside of the body.



from solid tumors in the bloodstream of cancer patients (35). Free DNA circulates in both healthy and diseased individuals. In control subjects, the mean concentration of soluble DNA in plasma has been estimated at 14–18 ng/ml, whereas in patients with different types of neoplasias, the mean concentration is 180–318 ng/ml. The increase in serum DNA in cancer patients might be mostly attributable to freely circulating tumor DNA. Therefore, serum/plasma is a compartment enriched in tumor DNA (35). There is much current effort using these approaches to detect either premalignant changes, cancer, or relapse of disease.

#### **Demethylating Agents as “Bio- or Chemosensitizers”**

More than 20 years ago it was shown that the methylation inhibitor 5-azacytidine was able to induce several differentiated cell phenotypes in an undifferentiated mouse embryo cell line

(36). It was later shown that 5-azacytidine and its deoxy version, 5-aza-2'-deoxycytidine, trap the DNA methyltransferase enzyme in a covalent complex with the DNA, resulting in a loss of DNA methylation with each round of cell division (37).

What makes this group of substances so interesting is the fact that they can reverse epigenetic changes and restore gene function to a cell. Treatment with DNA methylation inhibitors can restore the activities of dormant genes such as *p16* and decrease the growth rate of cancer cells in a heritable fashion (38). Genes that act as receptors for BRMs like *RAR-β2* (ligand is retinoic acid) or are involved in a cascade initiated by BRM toward cell death like *DAP-kinase* or *p14* (initiating cytokine is *IFN-γ*) have also been shown to be re-expressed by demethylating agents (9, 10, 12). Kwong *et al.* (1) demonstrated that beside other genes, *RAR-β2*, *DAPK*, and *p14* were methylated in

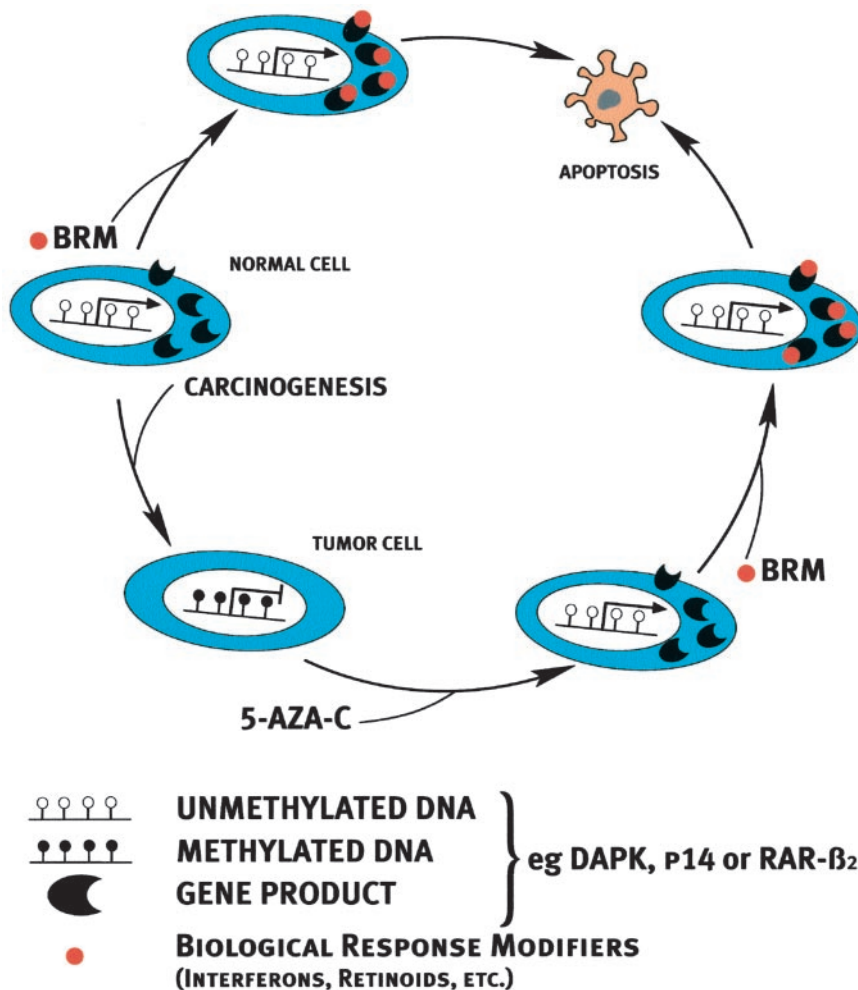


Fig. 2 Model as to how demethylating agents could be used as “biosensitizers.” Normal cells are expressing gene products that are relevant for the induction of apoptosis because of physiologically present biological response modifiers. During carcinogenesis, expression of such gene products can be silenced by aberrant promoter CpG island methylation. Treatment with 5-aza-2′-deoxycytidine (5-AZA-C) might demethylate the promoter region of these genes. The re-expressed gene products might serve as a target for biological response modifiers (administered in pharmacological doses), leading to subsequent cell death.

80%, 76%, and 20%, respectively, in nasopharyngeal carcinomas. Both groups of substances, IFNs as well as retinoids, alone or in combination, are active to varying degree in a number of malignancies (39) and are used in chemopreventive trials including those for premalignant lesions of the upper aerodigestive tract (40). 5-Aza-2′-deoxycytidine has already been used in clinical trials (41).

Hypermethylation of *caspase-8*, a key determinant of sensitivity for apoptosis induced by death-inducing ligands (tumor necrosis factor- $\alpha$ , tumor necrosis factor-related apoptosis-inducing ligand) or cytotoxic drugs (doxorubicin, cisplatin), was linked to reduced expression of this gene. Demethylation and, therefore, re-expression of caspase-8 restored sensitivity for chemotherapy- and death receptor-induced apoptosis (11).

In summary, this accumulating evidence might serve as a basis for a new therapeutic approach placing 5-aza-2′-deoxycytidine in a role as bio- or chemosensitizer, which can be applied systemically or locally (peritoneum, bladder, and so forth) followed by treatment with cytotoxic agents or biological response modifiers as outlined in Fig. 2.

**Future Directions.** One of the greatest tasks for researchers will be to use the new knowledge collected over the

past years to determine how dysregulation of one or more of the systems involved in DNA methylation during carcinogenesis can be prevented and how it can be reverted. On the other hand, this DNA alteration, which occurs during carcinogenesis and is also present in normal tissues as a function of age, can be used like a tag, marking a cell very likely to become a cancer cell or even a cancer cell itself. It remains to be seen whether DNA methylation can therefore be used as a marker for early detection, for a surrogate end point marker in chemoprevention trials, for a prognostic or predictive parameter in a patient with an invasive cancer, or as a marker that is able to indicate active disease after primary therapy.

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