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Review

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DNA Methylation in Cancer and Aging

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Abstract

DNA methylation is known to be abnormal in all forms of cancer, but it is not really understood how this occurs and what is its role in tumorigenesis. In this review, we take a wide view of this problem by analyzing the strategies involved in setting up normal DNA methylation patterns and understanding how this stable epigenetic mark works to prevent gene activation during development. Aberrant DNA methylation in cancer can be generated either prior to or following cell transformation through mutations. Increasing evidence suggests, however, that most methylation changes are generated in a programmed manner and occur in a subpopulation of tissue cells during normal aging, probably predisposing them for tumorigenesis. It is likely that this methylation contributes to the tumor state by inhibiting the plasticity of cell differentiation processes. *Cancer Res; 76(12); 3446–50. ©2016 AACR.*

Introduction

Cell identity is determined by two basic ingredients, its genetic makeup, together with epigenetic marks that determine how this information is read and utilized. In tumor cells, one can always detect alterations in the genetic material itself that include deletions, amplifications, or mutations, some of which appear to have a direct effect on growth-controlling functions and other properties characteristic of cancer (1). In addition, however, many stable epigenetic alterations are also associated with this disease, and these presumably work by changing the way normal intact genes are read in these same cells. In this review article, we have adopted a developmental approach for understanding what DNA methylation actually does, and this suggests new concepts for the role of this modification in tumorigenesis.

Programming of DNA Methylation

The DNA methylation pattern of the entire organism is generated in a programmed manner during normal development. Following fertilization, there is a step-wise process of erasure that removes most of the methyl groups derived from the gametic DNA, thus forming an epigenetic ground state. Then, at the time of implantation, there is a wave of *de novo* methylation that modifies almost all CpGs in the genome except for CpG islands that are protected (2). Although the exact mechanism of this process has not been elucidated, it appears that almost all islands are associated with RNA polymerase binding and the presence of histone H3K4me3 (3), suggesting that these regions may be marked for protection from *de novo* methylation simply by the presence of the transcription machinery itself at these sites. Furthermore, analysis of wild-type implantation stage embryonic stem cells in culture shows that over 98% of the methylated genes are also inactive even in embryonic stem cells that are completely lacking DNA methylation (4). Taken together, these observations indicate that the wave of global *de novo* methylation in the early embryo does not serve to actually repress previously transcribed genes, but rather helps maintain their continued silencing in somatic tissues that might otherwise be permissive to these genes.

Following implantation, changes in methylation take place in a site-specific manner and can involve either *de novo* methylation of genes that undergo repression during various stages of development or demethylation of tissue-designated genes. These targeted events are all mediated through cis-acting sequences and trans-acting factors that presumably recruit the molecular machinery required for altering the methylation pattern (2). In the case of silencing, the methylation event is always secondary to transcriptional inactivation and heterochromatinization, again suggesting that DNA modification itself does not actually cause repression. Once these epigenetic changes are made, they are automatically maintained even in the absence of the original initiating factors (5).

DNA Methylation in Cancer

There are two types of general changes in DNA methylation that appear to occur in a tumor as compared with normal cells of the same tissue type: demethylation within many regions of the genome in coordination with *de novo* methylation of select CpG islands. Much of the hypomethylation is concentrated within broad late-replicating Lamin-associated domains that make up about 40% of the genome and contain many repetitive sequences (6) while being relatively gene poor. More striking, however, is the modification that occurs on a wide range of CpG islands that are usually unmethylated in every tissue. Despite early observations suggesting that this occurs mainly at promoters of tumor suppressor genes and is a result of growth selection, it now appears that this is a widespread programmed process that may be based on a relatively universal mechanism mediated by polycomb-complex targeting (7–9). Although there are over 13,000 constitutively unmethylated CpG islands in the human genome, approximately 2,000 of these are marked with polycomb, a

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protein complex that operates as a repressor by bringing about local heterochromatinization. In tumors, this complex appears to be responsible for recruiting the *de novo* methylases, DNMT3A, and 3B (9, 10) that probably bring about the abnormal modification seen at these sites.

When Do Methylation Changes Occur?

Although the actual source of abnormal methylation or the trigger that initiates this process is not known, one possibility is that it comes about as a function of aging (11). Low-level *de novo* methylation of CpG islands is known to take place in normal tissues and has been shown to increase with age (6, 11, 12). In a similar manner, it has been shown that many of the general demethylation events observed in tumors also take place in normal cells (13), and this can even be seen in aging hematopoietic stem cells (HSC) and epidermal stem cells *in vivo* (14, 15). It is very possible that both of these changes are actually interconnected and result from a compartmental redistribution of the methylase complex from the replication machinery to select CpG islands (16). Taken together, these observations suggest that the overall aberrant methylation patterns commonly detected in cancer may have already been present to a large degree in the normal cell prior to its transformation.

Because most of the *de novo*–targeted islands are constitutively marked by polycomb, the resulting methylation pattern is similar in all tissue types, although the overall intensity of modification may vary widely between different tumors, with methylation being at its highest in colon cancer, for example, while being very low in brain tumors (17). Thus, in principle, there must be other factors that control this phenomenon *in vivo*, most of which are probably related to the specific developmental history of each primary cell type (18). It is well established that local inflammation can predispose tissues to cancer, and it is very likely that DNA methylation is also involved in this process (19). It has been shown, for example, that ulcerative colitis is characterized by changes in DNA methylation very similar to those ultimately seen in colonic tumors, and the same appears to be true in mouse models of chronic inflammation in various tissues (20). Another "environmental" factor that may influence the generation of abnormal DNA methylation patterns is oxygen stress, which has been shown to directly affect the recruitment of DNA methyltransferases to polycomb target genes, thereby promoting a tumor-like methylation profile (16).

Using high-throughput technology to detect mutations in cancer, it has been demonstrated that many of the affected genes are involved in DNA methylation metabolism or in the control of chromatin structure (1), raising the possibility that the epigenetic state of tumors may just reflect one consequence of the many mutations that occur in cancer. Although it is certainly likely that these genetic alterations can influence DNA methylation, it should be kept in mind that the underlying process of *de novo* modification in cancer is a fixed feature of tumorigenesis that is programmed as part of aging independently of gene mutations (21) and appears to take place prior to transformation. Nonetheless, these modifier gene mutations may certainly contribute to downstream posttransformation changes in DNA methylation patterns in a tumor-specific manner. Indeed, the cancer genome reveals many local foci of *de novo* and demethylation events that appear to encompass putative enhancer sequences harboring transcription factor–binding domains, and these changes are correlated with the dysregulation of nearby genes, many of which may be cancer promoting (22). In addition, tumor suppressor genes that do not appear to be targets for premethylation in normal tissues may still undergo later *de novo* modification in a percentage of tumors (23).

What Does *De Novo* Methylation Do in Cancer?

Because DNA methylation represents a molecular mechanism associated with gene repression, it has been assumed that *de novo* modification in cancer may contribute to the tumor phenotype by inhibiting genes that were initially active in the source tissue (24), especially those involved in tumor suppression. Although examples of oncogene-directed methylation-associated gene-repression pathways of this nature have indeed been observed in cancer cell lines (25), recent studies suggest that this viewpoint may be too simplistic. Several experiments have shown, for instance, that over 90% of genes that undergo *de novo* methylation in cancer are already transcriptionally repressed in the normal tissue (24), consistent with the observation that almost all of the target genes are binding sites for the polycomb complex (7–9). Indeed, these constitutively unmethylated genes are generally repressed from early embryogenesis through a process that utilizes EZH2 to methylate local histone H3 at the lysine 27 position, and it is probably this local heterochromatinization that brings about gene repression.

Many of these target genes are involved in development and differentiation, and while they are repressed during early embryonic stages, they can be activated when needed by removal of the polycomb complex (26). Because it is these exact same sites that undergo *de novo* methylation in cancer, it is not surprising that these genes are already repressed in the normal tissue prior to transformation. This modification may also inhibit upregulation of genes that would normally combat the tumor phenotype through processes like DNA repair or apoptosis, many of which are already classified as tumor repressors (27). It thus appears that cancer-associated *de novo* DNA methylation, rather than bringing about repression of active genes, is occurring mostly on silent genes, and if it has any influence at all, it would have to be in preventing their activation (28).

There is a considerable amount of evidence indicating that *de novo* methylation plays a role in tumorigenesis, mostly based on the use of 5-azaC, a compound that has the potential to cause hypomethylation. When tumor cells grown in culture are treated with 5-azaC, for example, a number of key *de novo* methylated genes were shown to undergo activation (29) and these cells often lose many of their cancer-like properties. As predicted, most of these are well-known polycomb targets (9). Even more convincing are experiments showing the effect of inhibiting methylation in mouse models of cancer. Using *min*$^-$ mice, it was demonstrated that weekly treatment with low doses of 5-azaC from birth almost completely prevents the appearance of intestinal adenomas in 3- to 5-month-old animals (30).

In the *min*$^-$ model, the generation of adenomas takes place in a two-step process. Although these animals are heterozygous for the min gene, spontaneous deletions or inactivation of the second allele is a frequent event, and this leads to the formation of local microadenomas (aberrant crypts in humans). Only approximately 1 in 20 of these ultimately form full adenomas (31). Treatment with 5-azaC does not have any effect on the appearance of

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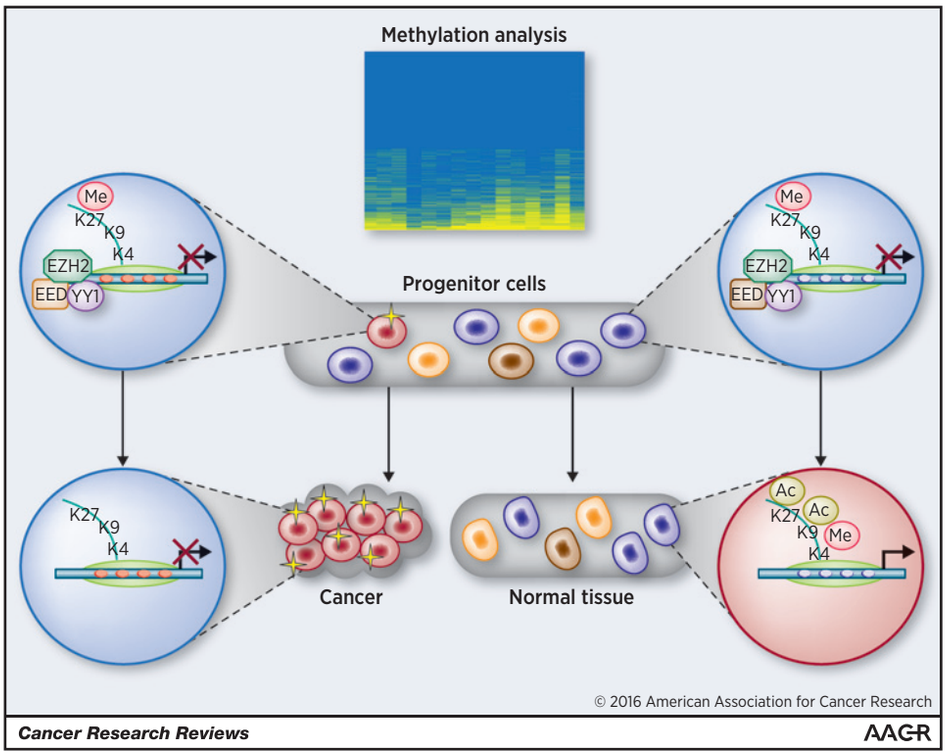

Figure 1.

In this proposed model, aberrant DNA methylation takes place at high levels in some (red) and at lower levels in other (brown, yellow, and white) progenitor cells. This skewed distribution can be visualized by carrying out single-molecule bisulfite analysis on DNA from normal tissue showing over 2,000 molecules of a sample island carrying 15 individual CpG residues (yellow, methylated; blue, unmethylated). While tumor-initiating mutations (+) can occur in any cell, those with high levels of methylation are predisposed to cancer formation. According to this scheme, most of the *de novo* methylation seen in cancer actually occurs prior to transformation and is then clonally maintained in the tumor together with the mutation. At the molecular level, polycomb target genes in normal progenitor cells are bound by a repressive complex that contains the histone methyltransferase EZH2 and other proteins. Although the expression of these genes is inhibited, most of them have unmethylated (white circles) CpG island promoters and can therefore become activated by removal of the repressor and alteration of local histone marks during differentiation to the mature cell type (right). In contrast, in cancer-prone progenitor cells, polycomb target genes have already become *de novo* methylated (red circles) during aging. Although these genes are able to shed their polycomb complex as the cells progress, the presence of DNA methylation does not allow them to become activated, thus preventing these cells from terminal differentiation and leaving them in a relatively proliferative state.

microadenomas. This suggests that while the first step in the tumorigenesis process is caused by the absence of the tumor suppressor (*APC* in man), the second step, expansion to an adenoma, requires DNA methylation. Interestingly, initiation of the treatment at 3 months of age did not affect the appearance of tumors (30), suggesting that the methylation required for tumor expansion occurs early, most probably before the transformation event itself.

Methylation and Tumorigenesis

Considering all the new data in the field of DNA methylation, it may now be possible to propose a model for how this modification can influence tumorigenesis. The findings on DNA methylation in cancer can be interpreted in two different ways. On the one hand, it is possible that normal cells become transformed through the occurrence of driver mutations and then undergo *de novo* and demethylation as a result of this event, setting in motion a series of programmed changes in gene expression. Alternatively, a subpopulation of normal cells that have already undergone changes in methylation, perhaps as a result of aging, may represent preferred targets for oncogenic transformation (11, 19). According to this second proposal, the presence of abnormal methylation in cancer actually comes about through selection of pre-existing normal cells characterized by a methylator phenotype (Fig. 1). Once this is formed, it would, of course, be preserved in progeny cells, much in the same manner as mutations.

There is a great deal of evidence that the DNA methylation seen in cancer and aging may stem from a small population of cells. Not only are the target sites found partially methylated in normal tissues, but are also highly modified in polyps (17), a very early stage in the generation of colon cancer in man. Colon epithelium is formed from adult stem cells located in crypts, and this tissue undergoes rapid turnover. Thus, the methylation pattern measured in normal colon actually reflects what is present in the stem cells. Interestingly, although DNA methylation appears to be quite uniform on individual molecules derived from a single crypt, the levels of modification vary considerably between crypts (32). Indeed, recent studies on normal colon using single-molecule bisulfite sequencing indicated that for any particular CpG

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island, the level of abnormal *de novo* methylation is not uniform. Rather, some molecules have very high levels, whereas others are completely unmethylated, and the same was seen for other adult tissues, as well (17). This strongly suggests that in each tissue, there may be cells with very low amounts of *de novo* methylation, whereas others are highly methylated at these CpG islands, and it is very possible that it is these cells that are most prone to transformation and subsequent growth selection. According to this idea, the general *de novo* methylation seen in cancer may actually already exist in a subpopulation of "normal" cells prior to their transformation by gene mutation (Fig. 1).

These observations suggest a new model for understanding how DNA methylation may play an active role in tumorigenesis. Colon cancer may serve as an instructive model for elucidating this idea. The colon is a renewing tissue fed by stem cells that constantly produce new proliferative cells that climb up the crypt and at some point undergo differentiation to form the epithelium. Many of the genes involved in this step are polycomb targets (9) that are repressed throughout development and get activated by removal of the polycomb complex, thus allowing them to be transcribed and produce the protein factors that drive differentiation. This polycomb repression mechanism is based on protein–DNA interaction, which generates local facultative heterochromatin, a relatively plastic structure that can easily be reversed by releasing the complex itself from its binding sites on the DNA. It appears that during aging, a subpopulation of stem cells in the colon undergoes *de novo* methylation of target CpG islands, and this presumably generates small patches of tissue that carry an aberrant DNA methylation profile (32).

This modification probably induces a state of constitutive heterochromatin, which is not easily reversible. Thus, proliferative cells in the crypt that carry this mark may be able to remove the polycomb complex itself, but would not be capable of activating the critical differentiation genes, thereby inhibiting these cells from undergoing a transition to epithelium, thus leaving them in a relatively proliferative state. Although this might not be sufficient for generating a tumor, it could very well provide necessary background for cells that have undergone transformation either through prior genetic predisposition or by spontaneous mutation. This concept that certain cells accumulate DNA methylation during aging and then serve as preferred targets for the transformation process is supported by the observation that both polyps (17) and normal tissue surrounding the tumor (33) are highly methylated and by the experimental evidence showing that 5-azaC is only capable of preventing the formation of intestinal tumors in mice if it is administered from early in life. Furthermore, it explains why only a fraction of the min^{-} microadenomas progress to true tumors (30).

Hematopoietic malignancies represent another good example for how DNA methylation changes may contribute to tumor phenotype. Both myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) increase dramatically with age and are marked by impeded differentiation and a shift toward myeloid output (14). Interestingly, several studies have already established that hematopoietic stem cells from both mouse and man actually undergo a natural aging process whereby they increase in number while their lymphoid differentiation capacity is diminished, leading to a myeloid-dominant phenotype (34). Whole-genome bisulfite analysis indicates that this aging process is associated with typical *de novo* and demethylation events normally found in tumors (6, 14), and there is a good possibility that these alterations in methylation actually play a role in promoting stem cell renewal and inhibiting differentiation. Strikingly, the changes in DNA methylation seen in aging stem cells are very similar to those detected in MDS (14), strongly suggesting that a large part of the "abnormal" methylation profile seen in this disease probably comes about as a natural result of aging even in the absence of genome mutations. Furthermore, these changes not only occur early, but also seem to work by inhibiting differentiation.

Conclusions

We have proposed a general model for the role of abnormal *de novo* DNA methylation in tumorigenesis, whereby relatively widespread methylation changes in a subpopulation of normal cells serve as a "driver" to prevent the activation of key genes required to induce terminal differentiation and its accompanying inhibition of cell proliferation. Although this represents an attractive hypothesis, it should be noted that there is as yet no definitive proof for this idea. To this end, it will be necessary to obtain additional evidence for this model in other tumor types and then to ultimately demonstrate that tumors are actually formed from cells that have abnormally high levels of CpG island *de novo* methylation. The most attractive aspect of this concept is that it is in keeping with the general role of DNA methylation *in vivo*, where at every point in development, this modification serves to prevent the reactivation of gene expression rather than being an inducer of repression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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