Methyl Deficiency, Alterations in Global Histone Modifications, and Carcinogenesis\textsuperscript{1,2}

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Abstract

The methyl-deficient model of endogenous hepatocarcinogenesis in rodents is unique in that dietary omission rather than the addition of chemical carcinogens leads to tumor formation. Thus, the biochemical and molecular events predisposing to cancer in this model result from chronic metabolic stress and provide an ideal model system to study progressive alterations that occur during carcinogenesis. Moreover, epigenetic alterations imposed by this diet are believed to be 1 of the main mechanisms responsible for malignant transformation of rat liver cells. In this study we examined the changes in global histone modification patterns in liver during hepatocarcinogenesis induced by methyl deficiency. Feeding animals the methyl-deficient diet (MDD) led to progressive loss of histone H4 lysine 20 trimethylation (H4K20me3), H3 lysine 9 trimethylation (H3K9me3), and histone H3 lysine 9 (H3K9ac) and histone H4 lysine 16 (H4K16ac) acetylation. A considerable decrease of H4K20me3 and H3K9ac was also detected in liver tumors induced by MDD. In contrast, liver tumors displayed an increase in H3K9me3 and H4K16ac. To determine the possible mechanism of alterations of histone modifications, we analyzed the expression of histone-modifying enzymes in liver during hepatocarcinogenesis. The expression of Suv4–20h2 and RIZ1 histone methyltransferases (HMTs) steadily decreased along with the development of liver tumors and reached its lowest level in tumor tissue, whereas the expression of Suv39-h1 HMT and histone acetyltransferase 1 (HAT1) substantially increased in tumors. These results illustrate the complexity and importance of histone modification changes in the etiology of hepatocarcinogenesis induced by MDD. J. Nutr. 137: 216S–222S, 2007.

Evidence accumulated in recent years shows that nutrition plays an important role in cancer etiology by inhibiting or enhancing carcinogenesis (1,2). Diet may contribute significantly to the causation of most human cancers (2,3). In this regard, uncovering the molecular mechanisms of action of dietary nutrients leading to neoplastic cell transformation is important for cancer prevention (1).

Deficiency of the major dietary sources of methyl groups—methionine, choline, folic acid, and vitamin B-12—is sufficient to induce liver tumor formation in male rats and certain mouse strains (4–7). The biochemical and molecular events predisposing to cancer in the methyl-deficient model of carcinogenesis may result from diet-induced chronic metabolic stress and provide an ideal model system to study progressive alterations induced by the sustained stress environment (7,8). Previous experiments of rodent methyl deficiency in vivo showed that such diets led to rapid fat accumulation in the liver, increased lipid peroxidation, necrotic and apoptotic cell death, increased cell proliferation, depletion of intracellular methyl group pools, an imbalance of deoxynucleotide pool resulting in uracil incorporation into DNA, DNA strand breakage, and increased genome-wide and gene-specific hypomethylation (7–14). All of these factors may contribute to the hepatocarcinogenic effects of the methyl-deficient diet (MDD).\textsuperscript{6} Moreover, the aberrant epigenetic alterations imposed by this diet may be 1 of the main mechanisms responsible for malignant transformation of rat liver cells (9,10,14–16).

In recent years the role of epigenetics in the etiology of human disease, including cancer, has been increasingly recognized (17–19). Epigenetic changes can be defined as changes induced in a cell that alter the expression of the information of the genome

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\textsuperscript{6} Abbreviations used: GST\textsubscript{\pi}, glutathione S-transferase \textsubscript{\pi}; H3K9ac, histone H3 lysine 9; H3K9me3, histone H3 lysine 9 trimethylation; H3S10ph, serine 10; H4K16ac, histone H4 lysine 16; H4K20me3, histone H4 lysine 20 trimethylation; HAT1, histone acetyltransferase 1; HMT, histone methyltransferase; MDD, methyl-deficient diet.
Histone H3 and H4 modifications and methyl deficiency

The results of previous studies of rodent methyl deficiency show clearly that MDD induces global and regional DNA hypomethylation in liver accompanied by altered gene expression (9,10,13–15,35), and these alterations are among the earliest key steps contributing to the carcinogenic effect of MDD (14,15,35). Development of liver tumors induced by methyl deficiency is characterized by 2 distinctive phases: transition from normal liver to a state of chronic liver injury (irreversible stage), followed by a second transition leading to the formation of liver tumors (irreversible stage) (15,35). The sequence of these pathological events is remarkably similar to the development of hepatocellular carcinoma in humans (35). In consideration of the tight interaction among all cellular epigenetic components, especially the link between DNA methylation and histone modifications, as well as the recently emerging evidence of profound changes of histone modifications in cancer cells (23,28,32), we examined alterations of global histone H4 and histone H3 modifications in liver during methyl deficiency in rats.

Figure 1 shows that feeding MDD resulted in rapid, substantial, and progressive decrease of H3 lysine 9 trimethylation (H3K9me3), histone H4 lysine 20 trimethylation (H4K20me3), and histone H3 lysine 9 acetylation (H3K9ac) and an increase of H3 phosphorylation at serine 10 (H3S10ph) in liver during the early stages of hepatocarcinogenesis (0–18 wk of deficiency). Previous studies showed that this initial transition period from normal liver to a state of chronic liver injury is characterized by intense apoptotic cell death, increased compensatory cell proliferation in liver, accumulation of DNA lesions, elevated DNA repair processes, and increased gene expression (12,35). Changes in histone H3K9me3, H4R20me3, H3K9ac, and H3S10ph during this period may simply reflect the various pathological events occurring in the liver during this transition phase. Similar observations were found in response to various environmental factors that induced cellular stress, such as hypoxia, chemical exposure (nickel chloride, tamoxifen), and exposure to ionizing radiation (36–39). During this stage of deficiency, the most notable changes were detected in H3K9me3 status.

Several possible explanations exist for the mechanism of loss of trimethylation at H3K9 and H4K20 after exposure to MDD during this period. First, because the methylation of chromatin at H3K9 is intimately linked to the methylation of DNA (33,40,41), the loss of H3K9me3 may be affected by loss of DNA methylation induced by methyl deficiency (14–16) and associated with global DNA hypomethylation. Second, the loss of H3K9me3 may be related to altered activity or expression of histone methyltransferases (HMTs) (42–44) or to active demethylation by recently discovered lysine trimethyl-specific histone demethylases JMJD2a and JMJD2b (45,46). Third, increased cell proliferation could also lead to loss of H3K9 and H4K20 trimethylation. Despite the importance of delineating mechanisms leading to loss of trimethylation at H3K9 and H4K20, the significance of defining the biological consequences of this loss is much higher.

One of the primary functions of H3K9me3 and H4K20me3 is the formation of constitutive heterochromatin and inhibition of transcription (47–49). The continuing substantial loss of H3K9 and H4K20 trimethylation could result in a more “relaxed” heterochromatin organization, which markedly impairs genome stability (47–49). Furthermore, trimethylation of H4K20 was recently shown to be crucial in the damage checkpoint control, and loss of histone H4K20me3 compromises the ability of cells to maintain cell cycle arrest (50). Additionally, H3K9me3 is absolutely necessary for cells to enter the terminal differentiation

at the transcriptional, translational, or posttranslational level without change in DNA sequence (17,20). In normal cells epigenetic information is hereditarily maintained to preserve cellular identity. In contrast, cancer cells are characterized by profound alteration of epigenetic regulation (19,21–24). The overall disruption of the epigenetic landscape is the most common feature of all human tumors and includes alteration in cytosine DNA methylation and a characteristic histone modification pattern (25). For >20% the DNA methylation patterns of neoplastic cell lines have been recognized as being substantially altered compared with normal cells (19,21). Two types of changes in the DNA methylation pattern occur in cancer: global and regional loss of DNA methylation and hypermethylation of certain CpG island–specific genes associated with gene silencing (19,21,24). Genes involved in DNA methylation also are disrupted in cancer cells (26,27). DNA in euakaryotic cells is intimately associated with a family of small, basic histone proteins forming a highly ordered and condensed DNA-protein complex termed chromatin. Because of this chromatin structure, changes in DNA methylation in cancer cells are not isolated events; they occur in the context of more complex epigenetic deregulation (28).

Chromatin is the physiological template of the genetic information and is composed of DNA, histones, and other chromosomal proteins. The fundamental repeating unit of chromatin is the nucleosome octamer, which consists of 147 bp of DNA wrapped around 2 copies each of H2A, H2B, H3, and H4 histones (29). Histones are evolutionarily conserved proteins that have a globular carboxy-terminal domain critical to nucleosome formation and a flexible amino-terminal tail that protrudes from the nucleosome core and contacts adjacent nucleosomes in a higher-order structure. The amino-terminal tails of histones are subject to posttranslational covalent modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation (30), and multiple histone modifications may occur on a given histone tail (31). In recent years these posttranslational modifications of histone proteins have been recognized to affect chromatin structure, gene transcription, and maintenance of epigenetic information. Alterations in global histone modification patterns in cancer cells remained largely unknown because most studies have been focused only on changes of a particular histone modification at individual gene promoters (32). Recently, a first draft of a deviant histone modification signature in human cancer was attempted (28). Because of the many different types of histone modifications and types of cancer, drafting a universal cancer-specific picture of aberrant histone modifications applicable for all types of cancer is an extremely difficult and challenging task. The complexity of this goal may be illustrated by histone methylation alone. Presently, there are 24 known sites of methylation on histones: 17 are lysine residues, and 7 are arginine residues (33).

Even these numbers, without taking into consideration the possible methylation states of lysine and arginine (mono-, di-, or trimethylated states), allow $3 \times 10^3$ distinct methylation variants of histones, which highlights the enormity of this task (33). Methylation at these sites has been linked to X-chromosome inactivation, heterochromatin formation, and transcriptional activation and repression (34), demonstrating a widespread role of histone methylation in various aspects of chromatin biology. Despite this fact, illustrating the importance of histone modifications in the maintenance of cellular epigenetic integrity and emerging evidence that aberrant histone modifications are main feature of tumor cells, the role of histone modifications in neoplastic cell transformation is largely unknown.
pathway (51); the loss of H3K9me3 compromises the balance between cell proliferation and differentiation processes, favoring cell proliferation in this case. Any or all of these mechanisms may promote neoplastic cell transformation. Indeed, we showed that a period between 9 and 18 wk of deficiency is characterized by development of irreversible changes in liver leading to tumor development (15).

A different trend in histone modification changes were observed during the second transition phase resulting in formation of liver tumors (Fig. 1). Development of liver tumors is characterized by the continuing progressive decrease in H4K20me3 during tumorigenesis, reaching its lowest level when tumors formed. In contrast H3K9me3 gradually increased after 36 wk of deficiency, reaching control levels in liver tumors. Substantial loss of H4K20me3 in liver tumors could severely impair viability of cells by altering chromatin structure (47–49). Therefore, the increased global trimethylation at histone H3-Lys9 in liver tumors may be a cellular defense mechanism for maintenance of heterochromatin organization and safeguarding the viability of cancer cells. In contrast to observed changes in H3K9me3 and H4K20me3, we have not detected substantial changes in mono- or dimethylation of H3K9 and H4K20. The reason for this may be related to different repeat-associated histone methylation states. Trimethylated H3K9 and H4K20 are associated with pericentric heterochromatin whereas monomethylated H4K20 and H3K9 are enriched within regions of chromosome arms (52).

The amino-terminal tail of histones is subject to multiple histone modifications that may occur within a short stretch of amino acid residues or even on the same amino acid residue (42–44). The occurrence or alteration of 1 modification may affect the subsequent addition or the function of other modifications (42,44). For example, methylation of H3K9 inhibits acetylation of H3K9 and phosphorylation of H3S10 whereas phosphorylation of H3S10, in turn, affects methylation, acetylation, or both of H3K9 (42). Similarly, methylation of H4K20 will affect H4K16 acetylation on histone H4 (44). Indeed, our data show that alterations in H3K9me3 and H4K20me3 induced by methyl deficiency were accompanied by multiple interdependent changes in other histone modifications. These changes may lead to aberrant nuclear architectures of “heterochromatic” subdomains in liver cells affecting many different cellular pathways; a recent study indicates that such changes have been associated with tumor progression (43).

Expression of histone-modifying enzymes and methyl deficiency

To determine possible mechanisms leading to alterations of histone modification, we analyzed the protein expression of histone-modifying enzymes in liver tissue during MDD-induced hepatocarcinogenesis. Expression of Suv4–20h2, PRDM2/RIZ1, and hPR-SET7 HMTs steadily decreased along with development of liver tumors and reached their lowest level of expression in tumor tissue (Fig. 2). A different trend was observed for the expression of Suv39h1 HMT and histone acetyltransferase 1 (HAT1). The lowest level of expression of these enzymes was detected in liver tissue after 36 wk of deficiency, followed by the increase in their expression in tumors. Results of recent experiments demonstrated that Suv4–20h2 HMT and Suv39h1 HMT are the main contributors to trimethylation of H4K20 and H3K9, respectively (43,47–49). In addition to methylation of histone

Figure 1 Western blot analysis of histone H3 and H4 modifications in liver of control rats and rats fed methyl-deficient diet. Acid extracts of total histones were separated by SDS-PAGE and subjected to immunoblotting using primary antibodies against H3K9me3, H3K9me2, H3K9me1, H3K9 ac, and H3S10ph (A) and H4K20me3, H4K20me2, H4K20me1, and H4K16 ac (B), respectively. All antibodies were obtained from Upstate. Equal sample loading was confirmed by Coomassie Blue staining and by immunostaining against histones H3 and H4. Consistent results were obtained in 2 independent experiments for each antibody. Representative Western immunoblot images are shown. Results are presented as change relative to age-matched control rats. * Significantly different from control at the same (n = 5, means ± SEM).
H3K9, Suv39h1 HMT may also be involved in the recruitment of Suv4–20h2 HMT to pericentromeric regions (47). Emerging evidence suggests an important role of Suv39h1 and Suv4–20h2 HMTs in the maintenance of genomic stability by maintaining stringent heterochromatin higher-order structure (47–50,53). The major phenotype observed in mice with genetically inactivated Suv39h1 or Suv4–20h2 HMTs is genomic instability in various somatic cells (53) associated with severe impaired viability of somatic cells and increased tumor risk (53,54). Suv39h-deficient mice display a specifically increased risk for late-onset B cell lymphomas (53). Furthermore, deficiency of Suv39h1 histone methyltransferase remarkably accelerates the development of lethal tumors in response to oncogenic Ras (54).

RIZ1 is another H3-K9 methyltransferase. RIZ contains the canonical retinoblastoma-binding motif LXCXE, nuclear hormone receptor binding motif LXXLL and PR domain, which shows the similarity to the catalytic motif or the SET domain of histone- or protein-methyltransferase (55). The retinoblastoma protein–interacting zinc finger gene RIZ expresses 2 products: RIZ1, which contains the PR domain and has tumor suppressive properties, and RIZ2, which lacks the domain and has no tumor suppressive properties (55). RIZ1 gene is often inactivated by mutations (55) or by cytotoxic promoter hypermethylation (56) in different types of human cancers, including colon cancer, liver cancer, and leukemia (56–61). RIZ1-deficient mice develop B cell lymphomas similar to those in Suv39h1 double-null mice (60). Recent findings show that mutations in RIZ1 gene reduce or abolish the H3-K9 methylase activity of the protein, implying an important role for this HMT activity in tumor suppression (55).

Another interesting finding of this study is the alteration of histone acetyltransferase expression in liver during methyl deficiency, which suggests a tumor suppressor property for HAT1. Together, the presented data suggest that the Suv39h1, Suv4–20h2, and RIZ1 HMTs and HAT1 could act as tumor suppressors by maintaining a methylation state at H3-K9 and H4-K20 and an acetylation state at H3 and H4, preserving the heterochromatin organization. Inhibition of their expression and activity may play a crucial role in neoplastic cell transformation. On the other hand, considering the crucial role of histone methylation and acetylation processes for the maintenance of chromatin structure and viability of cells, elevated expression of Suv39h1 HMT and HAT1 in liver tumors may be considered a cellular defense mechanism for maintenance of heterochromatin organization and cell viability. Overexpression of HAT1 and its concomitant aberrant histone acetylation was observed in a variety of cancers (61). In addition, increased expression of Suv39h1 HMT and HAT1 can assist in tumor progression by controlling the choice between growth and differentiation at later stages of tumor development.
Altering histone modification and carcinogenesis

Emerging evidence suggests that perturbations in covalent modifications of histones may not only be an important feature of cancer cells but may be implicated in the development of neoplasia (61). However, the altered epigenetic landscape of cancer cells by itself is not sufficient for conclusive answers about the role of these changes in carcinogenesis. To prove that alterations in histone modifications play a causative role in cancer initiation and progression, it is necessary to provide evidence for changes that occur at preneoplastic stages being present during later stages of cancer, additional changes occurring during tumor progression, and a mechanistic link between the changes and initiation and progression (7). The nature of changes in histone modifications and in expression of histone-modifying enzymes induced by MDD corresponds to the above parameters. For any induced changes detected at preneoplastic stages of carcinogenesis to be considered as neoplastic, they need to be stable and persist after the factor inducing those changes has been removed. To determine the possible role of alterations of histone modifications in carcinogenesis, we evaluated the evolution of histone modification changes in liver after refeeding methyl-deficient rats a diet with an adequate level of methyl donors. Only this approach clearly allows discrimination between carcinogenic and noncarcinogenic changes because only changes that persist after removal of a carcinogen are regarded as preneoplastic or neoplastic (62).

In rats maintained on MDD for 18 wk followed by feeding a methyl-adequate diet with a sufficient content of methionine, choline, and folic acid for 36 wk, liver tissue was characterized by the high incidence of hyperplastic nodules and by the persistence of enzyme-altered (glutathione S-transferase \( \pi \) [GST\( \pi \)]-positive) nodules, a sustained decrease in trimethylation at H3K9 and H4K20, and a reduced level of Suv4–20h1 and RIZ1 (Fig. 3). Accumulated evidence suggests that increased expression of GST\( \pi \) is a sensitive marker for initiated cells and represents precursor lesions, which are causally related to carcinogenesis in liver. The lengthened retention of GST\( \pi \)-positive foci, decreased H3K9 and H4K20 trimethylation, and decreased expression of Su4–20h1 and RIZ1 in liver after withdrawal of MDD indicates that these changes are directly associated with carcinogenesis.

Genetic determinants of the histone-modification pattern

In recent years the mouse has become the predominant animal model for many aspects of biomedical research, including nutrition and cancer in the postgenome era. The striking similarity between mouse and human biology provides a unique opportunity for studies focusing on delineating molecular mechanisms of action of dietary nutrients leading to cancer formation and prevention, which frequently are impractical and in most cases unethical to conduct in humans (63). Although the mouse models play a crucial role in our understanding of the genetics and pathophysiology of human disease, the relation between genetics and epigenetics has been mainly overlooked. In this study we have compared histone modification patterns in 6 different inbred mouse strains: DBA/2J, BALB/cJ, AKR/J, A/J, C3H/HeJ, and C57BL/6J. The most dramatic difference in histone modifications, especially in H3K9me3 and H3K9ac patterns, was detected in liver of DBA/2J and AKR/J mice (Fig. 4). These 2 strains of mice displayed much lower levels of trimethylation and acetylation at H3K9 than did BALB/cJ, A/J, C3H/HeJ, and C57BL/6J mice. Additionally, C3H/HeJ mice are characterized by decreased level of H4K20me3. As we mentioned earlier, low levels of H3K9me3 and H4K20me3 may be a predisposing factor for the tumor development (53). If this suggestion is correct, these 3 strains of mice should display higher susceptibility to tumor development. Indeed, DBA/2J and C3H/HeJ mice are characterized by a higher susceptibility to hepatocarcinogenesis than C57BL/6J mice (64–67) and AKR/J mice are characterized by a higher incidence of spontaneous or chemically induced thymic lymphoma (68). These data illustrate an intimate link between genetics and epigenetics and the importance of genetics as a predetermining factor of an individual epigenetic landscape, which may be a decisive factor in the cancer prevention strategy.

Conclusion and future research

The results of this study provided evidence of the importance of altered histone modifications and expression of histone-modifying enzymes in the etiology of hepatocarcinogenesis and a mechanistic basis by which these changes may contribute to the initiation and promotion of cancer. The reversibility of epigenetic events offers a target for chemoprevention of cancer by dietary intervention. However, the results demonstrated clearly that the existence of stage-dependent effects and genetic background should be taken into account in cancer prevention strategy. In view of these results, further studies will focus on providing experimental evidence for higher susceptibility of mouse strains with lower levels of histone H3 and H4 trimethylation (DBA/2J.

Figure 4 Western blot analysis of histones H3K9 and H4K20 modifications in liver of DBA/2J, BALB/cJ, AKR/J, A/J, C3H/HeJ, and C57BL/6J mouse strains. (A) Acid extracts of total histones were separated by SDS-PAGE and subjected to immunoblotting using specific antibodies against H3K9me3, H3K9ac, H4K20me3, and H4K16 ac. Representative Western immunoblot images are shown. These results were reproduced in 2 independent experiments. (B) Quantitative analysis of the H3K9me3, H3K9ac, H4K20me3, and H4K16 ac in liver of DBA/2J, BALB/cJ, AKR/J, A/J, C3H/HeJ, and C57BL/6J mice. * Significantly different from C57BL/6J mice \( n = 3 \), means \( \pm \) SEM.
and C3H/HeJ, as compared with C57BL/6J mice, to hepatocarcinogenesis induced by methyl deficiency. In addition, these studies will aim to provide information that early dietary supplementation with adequate levels of methyl group donors will prevent the development of liver tumors.

**Literature Cited**


