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Introduction: diet, epigenetic events and cancer prevention

Sharon A Ross,

Nutritional Science Research Group, Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Johanna Dwyer,

Office of Dietary Supplements, Office of the Director, National Institutes of Health, Bethesda, Maryland, and Jean Mayer Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts, USA

Asad Umar,

Gastrointestinal and Other Cancers Research Group, Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Jacob Kagan,

Cancer Biomarkers Research Group, Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Mukesh Verma,

Host Susceptibility Factors Branch, Epidemiology and Genetics Research Program, Division of Cancer Control and Population Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Dana M Van Bommel, and

Cancer Prevention Fellow at the Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Barbara K Dunn

Basic Prevention Science Research Group, Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Epigenetics is defined as heritable changes in gene expression not associated with alterations in DNA sequence.¹ DNA methylation, histone modifications, chromatin remodeling factors, and noncoding regulatory RNAs are all known to be involved in epigenetic regulation of chromatin structure and gene activity. These epigenetic regulatory processes are considered critical components of normal development in cellular differentiation, organogenesis, and aging. Additionally, there is mounting evidence that epigenetic abnormalities are causative factors in several diseases, including cancer. Evidence also continues to surface for constituents in food and dietary supplements to influence gene expression, as well as an individual's risk of developing some cancers. Since epigenetic regulatory processes may be susceptible to changes by environmental factors, they offer potential mechanistic explanations for how diet may modify cancer risk and tumor behavior.

The Division of Cancer Prevention (DCP), National Cancer Institute (NCI), and the Office of Dietary Supplements (ODS), Office of the Director, National Institutes of Health (NIH), hosted the symposium titled *Diet, Epigenetic Events, and Cancer Prevention*, on September

26–27, 2007. The objectives of the symposium were 1) to identify voids that are impeding progress in basic, translational, and clinical research related to the use of bioactive food components (BFCs) in cancer prevention, and 2) to provide information about new tools to facilitate epigenetic research. The 2007 symposium represented a continuation of a previous trans-Department of Health and Human Services workshop on *Diet, DNA Methylation Processes and Health*, held in August, 2001. That workshop gave rise to a number of publications² as well as an NCI Funding Opportunity Announcement (as a Request for Applications) that led to the funding of 10 projects in collaboration with ODS. NCI continues to support research on diet, epigenetic events, and cancer prevention through active funding opportunities currently available in the NIH *Guide to Grants and Contracts* available at <http://grants.nih.gov/grants/guide/index.html>.

The *Diet, Epigenetic Events, and Cancer Prevention* symposium provided a critical synopsis of the strengths and weaknesses of the evidence linking diet and bioactive food components with epigenetic processes (including DNA methylation, histone modifications, chromatin remodeling factors, and non-coding regulatory RNAs), their implications for cancer prevention, as well as the next steps for advancing research encompassing the inter-related fields of diet, epigenetic events, and cancer prevention. Symposium topics included the following: critical windows of time for DNA methylation modifications and potential modification by dietary factors; analyzing DNA methylation patterns in human populations and intervention studies to assess relationships with diet; non-coding RNA in transcriptional gene silencing and evidence for diet and bioactive food components in modulating this gene expression regulatory mechanism; recent evidence suggesting an impact of BFCs on histone modification and gene expression; and a discussion of additional emerging issues and approaches in epigenetics research. These topics were addressed by speakers and during panel and group discussions. A brief summary of the presentations and panel discussions is provided here. The remaining articles in this supplement summarize the presentations and views of the speakers in the sequence they were given during the symposium.

Are there critical times of DNA methylation and potential modification by dietary factors?

The first four presentations focused on the evidence for dietary modulation of DNA methylation during various windows of susceptibility. Two discussed the viable yellow agouti (A^{vy}) mouse model³ that has been used to investigate the impact of nutritional and environmental influences on the fetal epigenome and subsequent phenotype. One presentation described the effects of maternal dietary supplementation with genistein, methyl donors and/or bisphenol A (an endocrine active compound commonly found in polycarbonate plastics) on coat-color phenotype and DNA methylation of the A^{vy} locus in offspring. The other agouti model presentation described the cumulative effects of maternal methyl donor supplementation over generations. Results from the F0 to F4 generations showed a trend for decline in the numbers of mice with yellow coats, raising the possibility that stable extinction of the viable yellow agouti (unmethylated) A^{vy} phenotype could occur through selection and dietary modification. Because in utero exposure to methyl donors did not increase the density of CpG methylation in either the F1 or F2 generations, it was suggested that other epigenetic mechanisms may be important in the shift to a silent state of the A^{vy} locus. Although the exact roles remain to be elucidated, the activities of histone modifications and non-coding RNAs were proposed as likely mechanisms mediating transgenerational epigenetic inheritance. Adult mouse models and cancer cell lines were also considered during this session. One speaker provided evidence from a mouse (C57B16 mice) study that examined the synergy between mild deficiencies in one-carbon nutrients, e.g., folate, vitamin B₂, vitamin B₆, vitamin B₁₂ (the elderly are among the most vulnerable to mild deficiencies of these nutrients), on *Wnt* signaling in colonic epithelium. The

induction of DNA strand breaks, rather than changes in DNA methylation, in the *Apc* gene was associated with a significant reduction in its expression in mice on the diet deficient in all four vitamins. In other studies, global and specific promoter methylation (*p16*) have been found to be modulated by folate availability in older, but not in younger, adult animals.

Evidence for the inhibitory effect of the green tea polyphenol epigallocatechin-3-gallate (EGCG) on DNA methyltransferase-1 (DNMT1) activity was presented. EGCG was also found to reverse CpG island hypermethylation and to reactivate methylation-silenced genes such as *p16^{INK4a}*, retinoic acid receptor β (RAR β), *O*⁶-methylguanine methyltransferase (MGMT), human mut-L homolog 1, and glutathione *S*-transferase- in several cancer cell lines. The effect of EGCG may be gene specific or cell line specific and was not as robust as the demethylating drug 5-aza-2'-deoxycytidine.

The experimental evidence presented in this session suggested that diet impacted epigenetic modulation during different times in the lifespan. Other topics discussed during this session included the amount of individual dietary constituent needed to bring about epigenetic modulation and subsequent phenotypic change, as well as whether there are regions of the epigenome that are more susceptible than others to dietary modifications. Many participants stressed the need to further our understanding of the interactions among diet, epigenetics, and crucial times for exposure during development and throughout the entire lifespan.

Examining diet and DNA methylation patterns in human population and intervention studies: what is needed to move forward?

Four speakers addressed the importance of characterizing methylation patterns in humans. The first speaker defined 'epigenetic epidemiology' as the study of the associations between epigenetic variation and risk of disease. The importance of applying such an approach to the problem of obesity was discussed. Maternal obesity during pregnancy and/or lactation has been hypothesized to induce epigenetic alterations that facilitate obesity in offspring. Such effects could have an impact on chronic disease later in life. Because there is a dearth of knowledge regarding the potential role of epigenetic mechanisms in body-weight regulation, it was proposed that extensive research in appropriate animal models is necessary to develop specific hypotheses that can be tested in epigenetic epidemiologic studies of human obesity.

Gene-promoter methylation in DNA from sputum was highlighted as a biomarker that could be important for early detection of lung cancer and monitoring response to preventive and treatment interventions. The studies that were described suggest that sputum can be used as a surrogate for tumor tissue to predict the methylation status of advanced lung cancer in situations where biopsy is not feasible. Goals of the ongoing Lung Cancer Prevention Study, a phase-III chemoprevention trial of selenium supplementation, were described. The investigators plan to determine the prevalence of methylation in an eight-gene panel from sputum and blood after tumor resection. Individuals with positive methylation markers will also be followed longitudinally to determine whether selenium alters their methylation profile.

The third presentation described a mathematical model based on the biochemical properties of folate-mediated one-carbon metabolism, which can be used to simulate gene-gene or gene-environment interactions, as well as specific experimental conditions. The model suggests that some of the long-range allosteric regulatory mechanisms have evolved to protect the cellular 'methylation rate'. The speaker described how new insights into the complex nature of folate and DNA methylation in carcinogenesis can be obtained by mathematical modeling, as well as how such modeling can provide mechanistic information and pilot data for targeted preclinical and clinical studies.

The final presentation in this session concerned the results from the Aspirin/Folate Polyp Prevention Study, which examined the effects of folate supplementation in participants with recent adenomas.⁴ Both global methylation and age-related CpG island methylation (in two gene promoters: ER α and SFRP1) in normal colonic mucosa were assessed in a study subgroup. Global methylation in colonic mucosa was found to vary little in this adult population at risk for colonic tumors, but gene-specific hypermethylation (at ER α and SFRP1) was found to be associated with age and location within the colon. No other factor, including dietary factors, was associated with hypermethylation at these sites. The speaker concluded that the effects of demographics, lifestyle factors, and behaviors (e.g., smoking, alcohol consumption, and physical activity), folate, diet, or methylene tetrahydrofolate reductase (MTHFR) genotype are likely to be smaller than currently measurable, if they exist at all.

The work presented in this session was encouraging for the development of DNA methylation markers as potential outcome and surrogate endpoint biomarkers in dietary intervention and epidemiological studies. It was suggested that those planning epidemiological studies should consider utilizing, when justified, cells/tissues other than white blood cells, such as sputum, oral buccal cells, exfoliated cells, and adipocytes as sources of DNA for epigenetic analysis. The need for high throughput technologies, particularly for clinical studies, was also discussed during this session. Additionally, the need to determine the effects of short-term dietary interventions versus long-term dietary intake on epigenetic markers was mentioned. The utility of global hypomethylation versus gene-specific hypermethylation for biomarkers was highlighted. More research on the strength of the evidence for histone modifications directing DNA methylation in mammalian cells as well as the relationship between one-carbon metabolism and promoter and global DNA methylation is needed. These topics are excellent candidates for further in-depth analysis and discussion leading to future research in cancer prevention.

What is the role of non-coding RNA in transcriptional gene silencing? Is there evidence that diet and bioactive food components modulate this gene expression regulatory mechanism?

Two speakers addressed non-coding RNA and its role in epigenetic processes, as well as its relationship to cancer. One presentation described the impact of non-coding RNA on the regulation of gene expression. Evidence was presented that endogenous small RNAs suppress gene expression at the transcriptional level in non-mammalian species. However, the specific details about the protein components of the small RNA transcriptional silencing complex, the temporal aspects of gene silencing, the detection of endogenous small RNAs that are operative in transcriptional modulation, as well as ability to direct stable, long-term gene silencing remain unresolved in mammalian models. The differential expression of microRNAs (miRNA) during hepatocarcinogenesis induced by methyl-deficiency in rats was also presented. Evidence was provided for early dysregulation of miRNA in the process of hepatocellular carcinogenesis, which could be reversed by restoring dietary methyl donors. These results suggest that miRNAs are potential biomarkers for early detection of pre-cancer and targets for chemoprevention. This session highlighted the need for further research to determine the role of non-coding RNAs in transcriptional gene silencing (vs. post-transcriptional gene silencing) and in cancer prevention. Research is also needed to identify the impact of bioactive food components on the regulation of microRNA and other noncoding RNAs.

How strong is the evidence for the impact of diet on histone modification and gene expression?

Four presentations focused on the influence of dietary constituents on histone modification and their subsequent effects on gene expression and/or cellular phenotype. In the first presentation, sulforaphane, a cancer-protective compound found in cruciferous vegetables, was reported to inhibit histone deacetylase (HDAC) activity using both in vitro and in vivo models. Most interesting was the finding that in healthy human subjects, a single ingestion of 68 g (1 cup) of broccoli sprouts inhibited HDAC activity in circulating peripheral blood mononuclear cells 3–6 h after consumption, with concomitant induction of histone H3 and H4 acetylation. Because of these findings, participants discussed whether eating large amounts of broccoli early in life could result in deleterious rather than beneficial effects. Although sulforaphane has been shown to selectively induce apoptosis in cancer cells but not in normal cells, additional research is warranted to determine beneficial versus deleterious responses to BFCs during vulnerable periods. The next presentation concerned the effect of diallyl disulfide (DADS) from garlic on histone acetylation status in vitro, in human tumor colon cell lines, and in a non-tumorigenic animal model. In the in vivo model, histone H4 and H3 acetylation was increased in isolated colonocytes following administration of DADS. Discussion centered on the need to perform a dose-response experiment because only a very high dose of DADS was utilized in the work presented. Interestingly, data analyzed from cDNA expression arrays suggested that DADS could modulate the expression of genes encoding proteins involved in several cellular processes including cell cycle regulation, proliferation, metabolism, detoxication, signal transduction, and protein transport. Evidence was also provided that reactive oxygen species can alter nuclear histone acetylation and deacetylation (chromatin remodeling) through induction of histone acetylase activity, leading to increased NF- κ B-dependent gene expression of proinflammatory mediators. Findings were also presented that dietary polyphenols (e.g., curcumin, resveratrol) can inhibit inflammation and restore glucocorticoid efficacy (which is lost under oxidative stress) through upregulation/restoration of HDAC2 activity in monocytes/macrophages. Also discussed was the finding that oxidative stress can inhibit HDAC activity and enhance inflammatory gene expression, leading to a chronic inflammatory response in disorders such as chronic obstructive pulmonary disease. Thus, there is an increasing awareness that HDACs are implicated in multiple disease conditions. An important area for future research will be to clarify the role of dietary regulation of HDACs in many diseases as well as during periods of vulnerability (development and aging).

The last presentation highlighted biotinylation sites on histones H2A, H3, and H4 at distinct lysine residues. Biotin deficiency was found to be associated with decreased abundance of biotinylated histones at transposable elements, increasing the transcriptional activity of endogenous retroviruses and genomic instability. The impact of histone biotinylation on gene repression using ChIP assays combined with DNA microarrays was also discussed.

Many participants indicated there is a need for technology development to better understand the mechanisms involved in biotinylation, acetylation, methylation, ubiquitination and sumoylation of histones and how HDACs regulate downstream pathways. It is also not clear how modifications of histones or HDACs alter DNA methylation patterns. Therefore, the sentiment was that future efforts should concentrate on this area to elucidate the association between histone modifications and regulation of DNA methylation. Other topics discussed during this session included the possible non-selective inhibition of multiple HDACs and the various pathways regulated downstream. Another concern was whether there is specificity of BFCs for particular HDACs that might be exploited to target HDACs for purposes related to the prevention or treatment of cancer and proinflammatory conditions. Additional points

raised in the discussion were whether genetic polymorphisms are involved in histone modification pathways and interactions, the need to explore whether normal cells can protect themselves against unwanted effects of BFCs, and whether dietary HDAC inhibitors act in a synergistic or additive manner with butyrate (a known HDAC inhibitor) formed in the gut.

What are the emerging issues and approaches in epigenetics research?

Six presentations addressed emerging issues by highlighting new tools, techniques, approaches, and models for epigenetic and/or epigenomic studies. Current applications of epigenomic tools were described. Evidence was presented for the use of chromatin immunoprecipitation (ChIP)-based approaches (e.g., ChIP- microarrays, ChIP- serial analysis of gene expression) to examine global epigenetic marks (referred to as epigenomics) in various cell systems. The implications for examining diet-related epigenomic alterations using these tools were discussed.

The second presentation described the perturbations in folate metabolism (S-adenosylmethionine synthesis (SAM) and/or deoxythymidylic acid synthesis (dTMP)) that are associated with risk for colon cancer. Evidence was presented for the involvement of cytoplasmic serine hydroxymethyltransferase (cSHMT) as a metabolic switch that directs the partitioning of folate-activated one-carbon units between dTMP and SAM as well as evidence for the importance of cSHMT activity in colon cancer. It was mentioned that gain-of-function and loss-of-function cSHMT mouse models are being developed to help elucidate the contributions of dTMP and cSHMT pathways in colon cancer.

Information was also provided about the use of a new mouse model, the MIN-O model, to study the influence of dietary folate (both excess and deficiency scenarios) on DNA methylation during the transformation of premalignant to malignant mammary lesions. These investigations are expected to help delineate the roles of folate and DNA methylation in mammary tumorigenesis and assist with the identification of epigenetic targets such as specific hypermethylated genes and associated methyl-CpG-binding proteins that could contribute to malignancy.

The impact of a dietary constituent (EGCG) on the chromatin remodeling factor, Bmi-1, which is a member of the polycomb repressive complex 1, was also reviewed. The functional role of Bmi-1 in squamous cell carcinoma cells was initially described. EGCG was found to suppress Bmi-1 levels and reduce Bmi-1 phosphorylation, resulting in displacement of the Bmi-1 polycomb protein complex from chromatin and reducing survival of transformed cells. Such studies suggest that dietary agents may reduce cancer cell survival by altering epigenetic control of gene expression.

Information was shared about the NIH Roadmap Epigenomics Program. Interested investigators are encouraged to visit the NIH Roadmap weblink (<http://nihroadmap.nih.gov/epigenomics/>) for more information about the program and available funding opportunities.

The final presentation of the symposium described a study that examined the inheritance pattern of epigenetic modifications in humans. Allele-specific chromatin modifications (i.e., of parental or maternal origin) were determined using an allele-specific ChIP-on-chip assay in tandem with the Affymetrix 10K single-nucleotide polymorphisms (SNP) chip. Application of this technique with principal component analysis resulted in the ability to cluster epigenetic differences between families. Additional studies on the inheritance patterns of epigenetic modifications may help enhance the understanding of families that have multiple cancers, which are currently unexplained by mutations in known cancer-causing genes. They may also assist in identifying epigenetic targets for directed

interventions (drug or dietary) to reverse cancer risk. Overall, this presentation raised considerable interest about the relationship between genetic-epigenetic interactions in cancer risk and prevention. In addition to describing new animal models and techniques for epigenetic/epigenomic research, the participants in this session, provided new approaches for examining and unraveling the relationship between diet and cancer prevention through an enhanced understanding of epigenetic markers and mechanisms.

Future research directions

During the meeting, a number of research areas and technology needs were identified as important for moving research forward on epigenetics, diet, and cancer prevention. Many of these recommendations are captured in statements below.

Research areas

1. Timing and exposure – When are BFCs beneficial and are there circumstances when they might be harmful?
 - a. Identify crucial times for exposure during development and throughout the lifespan
 - b. Evaluate if response is related to cancer stage, i.e., during initiation versus progression
 - c. Identify circumstances that dictate a beneficial or deleterious response
 - d. Assess histone deacetylase (HDAC) inhibitors and other epigenetic modulators in healthy people
2. Improved understanding of the biochemistry and effects of BFCs in epigenetic processes
 - a. Identify relevant BFC compounds and metabolites
 - b. Determine effective doses and concentrations
 - c. Identify relevant BFC targets
 - d. Summarize data on mechanism of action of BFCs
 - e. Ascertain pharmacokinetic data
 - f. Evaluate effective and safe delivery routes
3. BFCs effects on epigenetic machinery
 - a. Identify and characterize triggers of histone modifications and gene silencing
 - b. Examine specificity of BFCs for particular HDACs and histone acetylases (HATs)
4. Links between epigenetic events and changes in gene expression
 - a. Identify mechanism(s) by which DNA methylation changes are related to changes in gene expression (e.g., direct effects on target DNA or DNA methylation changes as a result of upstream events triggered by a BFC)
 - b. Elucidate mechanism(s) by which histone modifications affect gene expression

- c. Expand the understanding of the mechanisms involved in biotinylation, acetylation, methylation, and ubiquitination of histones
- d. Promote a greater understanding of the link between one-carbon metabolism or other pathways relevant to methyl metabolism and promoter methylation
- e. Clarify the hierarchy for methyltransferase activity and CpG site methylation
- f. Evaluate tissue-specific epigenetic effects
- g. Determine importance of genetic polymorphisms in enzymes involved in relevant metabolic pathways (e.g., methylene tetrahydrofolate reductase) that influence epigenetically controlled gene expression
- h. Identify epigenetic marks to use for early cancer detection and to monitor response to preventive or therapeutic interventions

Technology needs

1. Expand techniques for quantifying DNA methylation and histone modifications
2. Develop methods for genome-wide scans for DNA methylation and histone modifications
3. Create mathematical models to analyze how perturbation of one enzyme in a pathway affects downstream events and data to validate the models. Such models could be used to generate data for pilot studies and identify metabolites or nutrients with important effects that should be carefully monitored in trials.
4. Utilize technology for methyl-typing tumors
5. Develop robust multiplex assays, better high throughput assays, and commercialization of such assays
6. Construct more effective databases, computational approaches, and tools for integrating epigenetic data
7. Characterize reference epigenomes (profiles of DNA methylation and histone modifications)
8. Develop relevant animal and tissue culture models, including models that incorporate the effects of the microenvironment
9. Generate high-throughput assays applicable to screening large populations of diverse ethnicity and races

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