GENETIC AND EPIGENETIC CHANGES REGULATED BY BIOACTIVE MOLECULES IN CANCER THERAPEUTICS

by

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A DISSERTATION

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Cancer biogenesis stems from genetic and epigenetic changes that regulate various signaling pathways responsible for growth, division, and proliferation. Genetic changes occur through mutations that contribute to loss of function, overexpression or repression of proteins involved in metabolic pathways. The changes place individuals at risk to diseases, including cancer. However, epigenetics is a mechanism of gene control that occurs through changes in chromatin structure without alterations to the DNA sequence and is mostly reversible. Epigenetic alterations have been implicated in colorectal cancers (CRCs), transforming normal colonic epithelial cells. Aberrant DNA methylation and chromatin modifications have been shown to contribute to CRCs. These mechanisms alter genes that drive the initiation and progression of CRC. The novel approach of using diet-derived bioactive molecules that reverse the nature of epigenetic events that control gene expression important to apoptosis, differentiation or cell death pathways may serve as a treatment option for CRC. The aim of this dissertation was to determine the effectiveness of epigallocatechin-3-gallate (EGCG) and sodium butyrate (NaB) as epigenetic modulators against CRCs. Our investigation showed that the combination of EGCG and NaB was effective against CRCs by reducing cell viability and inhibiting colony formation, inducing apoptosis and cell cycle arrest in G2/M phase for p53-wild type (WT)-expressing RKO and HTC-116 and G1 phase for p53-mutant HT-29 CRC cells, decreasing survivin, a highly upregulated anti-apoptotic protein for all CRC cell
lines tested, inducing nuclear p21 in a p53-dependent manner in RKO CRC cells and inducing phosphorylated γ-H2AX a marker of DNA damage in RKO CRC cells. The EGCG and NaB combination also led to increased nuclear NF-κB-p65, a protein found to be associated with DNA damage in order to enhance repair mechanisms, in RKO CRCs, and a down-regulation of DNA methyltransferase1 (DNMT1) in all three CRC cell lines as well as decreased DNNMT3A and 3B levels and percent CpG methylation in RKO CRC cells. Further, HDAC activity was reduced in all cell lines tested. The levels of acetylated H3 level an epigenetic marker for gene expression was also observed in response to EGCG and NaB treatment in RKO CRC cells. Taken together, these findings demonstrate that EGCG and NaB are promising and effective chemotherapeutic bioactive molecules against colorectal cancers.

Keywords: Epigenetics, DNMTs, HDACs, EGCG, NaB, survivin
DEDICATION

I dedicate this dissertation to my dearest husband, Ivan. Your hours of single parenting provided the much needed hours for research and writing. To our wonderful son Aidan, thank you for being patient and understanding. You both have been my best cheerleaders.

To my loving mother Sophia and father Sylvester, a special feeling of gratitude. You have been great role models and pillars of support throughout my life. To my sister and my in-laws, your encouragement along the way is truly appreciated. Thank you for understanding my absence at your family functions and get-togethers.
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THE ROLE OF NUTRACEUTICALS IN CHEMOPREVENTION AND CHEMOTHERAPY AND THEIR CLINICAL OUTCOMES

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INTRODUCTION

Diet influences both the etiology and treatment of diseases. A diet high in saturated fats, cholesterol and sodium and low in fiber, protein, essential fat and vitamins can trigger imbalances in the micronutrient environment of the cell affecting cellular functions. Free radicals, the most common cell damaging molecules, stem from metabolic activity within the cell. Dietary foods such as vegetables and fruits that are rich in antioxidants quench free radicals and prevent DNA damage [1]. The chemical constituents of foods that provide such health benefits are made available through the digestive process and are called bioactive molecules and when used in disease treatments are termed as nutraceuticals [1]. Bioactive molecules from various food origins are now being used in preclinical and clinical trials against many diseases, including cancer [2]. Some of these molecules have similar activities, trigger similar pathways or genes, and some have different mechanistic actions. Because of their health promoting benefits, relative safety and ease of availability, bioactive molecules are considered suitable for treatment of cancers.

Initially, mutations in DNA were thought to be the primary cause of disease outcomes. However, epigenetics, a new mechanism of gene control, was introduced to the scientific world and with that the perception of gene regulation changed. It is now accepted that changes to the chromatin by reversal of chemical modifications influence gene expression. Of these, the most well-studied mechanisms are DNA methylation and histone modifications [3]. These epigenetic modifications induce euchromatin or heterochromatin states that determine gene expression. Enzymes that catalyze the
modifications control the dynamic equilibrium of the states through changes either on the DNA or histones. DNA methylation is specific to methylation of cytosine residues in eukaryotes [4]. However, histones can undergo several modifications such as acetylation, methylation, ubiquitination, sumoylation and phosphorylation each of which, or as a group, affects the outcome thereby adding another layer of complexity to epigenetic control [5].

Epigenetics plays a major role both in aging and age-associated diseases, including cancers of various origins [6]. Diet and the environment control these processes [7]. Studies have shown that dietary constituents of a mother can have a major impact on the health of her offspring and that of future generations contributed by a process known as transgenerational epigenetics [7]. A closer examination of the link between diet and cancer has shed light on the role of bioactive components in regulating epigenetic mechanisms. A majority of these molecules target epigenetic enzymes. DNA methyltransferases and histone acetylases/deacetylases target CpG dinucleotide residues and histone moieties, respectively [8]. DNA methyltransferase 1 (DNMT1) has maintenance methylation activities and requires a hemimethylated DNA template, whereas DNMT3A and DNMT3B are considered de novo methyltransferase, are required for new methylation patterns and act on nonmethylated substrates [8]. Other studies have shown that epigenetic enzymes could have co-epigenetic enzymatic properties [9]. In that, DNMT1 is a predominant methyltransferase but is also associated with deacetylase activity [9].

When epigenomes are altered, induction of genes that promote tumoriogenesis can result. Synthetic drugs that target epigenetic enzymes for therapeutic purposes have
been tested in clinical trials [10]. With synthetic drugs, the potential of destroying normal cells is relatively high resulting in various side effects. The novel approach of using bioactive molecules with epigenetic enzyme inhibitory properties in treatment is therefore pursued. Combination therapies have been more sought after than monotherapies as synergistic or additive property of the molecules may enhance the observed chemotherapeutic effect.

The commonly used and well-studied molecules with potent epigenetic effects have been described elsewhere [2]. Of these the polyphenol EGCG has been associated with DNMT1 inhibitory activity. Methylation activities of DNMT1 occur at cytosine residues at the C5- position and require $S$-adenosylmethionine (SAM) as the methyl donor [11]. Hypermethylation of CpG islands is usually associated with gene silencing [11]. It is thought that oncogenes may become hypomethylated and tumor suppressors become hypermethylated to enhance the tumorigenic phenotype [11]. EGCG functions by docking with the catalytic site of DNMT1 thereby preventing the enzyme from methylating the DNA [12]. Thus, treatment with EGCG can potentiate hypomethylated states. EGCG is highly unstable and therefore its efficacies require higher doses [2]. However, combining EGCG with other bioactive molecules with similar or different epigenetic potentials can enhance preventive or therapeutic effects [2].

Chromatin consists in part of nucleosomes that are octamers of histone subunits to which 146 bp of DNA are wrapped around [13]. The protruding tails of histones undergo various reversible modifications that change the conformation of the chromatin [13, 14]. The process, timing and cues for these modifications are still not well understood and involve a complex set of enzymatic proteins. Acetylation and
deacetylation of histones are the most analyzed and understood of the reversible histone modifications. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) govern these changes [13]. Molecules that target these enzymes alter the expression of genes that are induced in cancer formation [2, 14] . There are 18 HDACs identified in total in mammalian system and are divided into four major classes; Class I, II, class IV and sirtuins [10]. The specificity of HDAC inhibitors to the HDACs varies and depends on the HDAC and class to which it belongs. Drugs that target HDACs are classified into four major categories; hydroxamates, cyclic peptides, aliphatic acids and benzamides [15]. Of these categories, butyrate, an aliphatic HDAC inhibitor, is a dietary constituent and is also produced through the microbial fermentation of fiber [16, 17]. HDAC1 protects from DNA damage, sustains DNA damage checkpoint, maintains DNA replication, and regulates oxidative stress and non homologus end joining (NHEJ) [18]. A prodrug form of butyrate, tributyrin, has undergone clinical and pharmacological testing in advanced solid tumors and has been shown to be effective and well tolerated [19]. Combining the anti-neoplastic effects of individual bioactive molecules may thus prove more effective in treatment of cancer than single therapies. Combination therapies have been used against many cancers although combination therapies in colon cancer are only a few. The choice of the combination depends on its concentration at the site of treatment, the availability and source. EGCG and NaB reach the colon through the digestive process and NaB from dietary sources such as milk-fat and the fermentation of dietary fiber is present in the colon in millimolar concentrations. The effects of these molecules in the colon may have a profound effect in terms of treatment.
Colon cancer is the third leading cause of death in the US among both men and women and is commonly referred to as colorectal cancer (CRC). This includes the major portion of the large intestine, the colon, followed by the rectum. Although genetic and epigenetic mechanisms affect CRC development, diet and environmental factors also contribute to the development of CRCs [20]. A high fat diet predisposes individuals to a higher risk of CRCs [21, 22]. Among Asians, the rate of CRCs is much lowered, and a diet rich in fruits and vegetables may account for the observed lowered rates [23]. Dietary molecules that have health-promoting and cancer-preventing benefits are encouraged by many organizations including the American Cancer Society as a cancer preventive strategy [24].

CRCs develop through a multi-stage process, starting as polyps that are benign growths of the epithelial cells on the inner lining of the colon and rectum. Increased mutations or chromatin changes in these polyps can lead to neoplastic transformation. Although mutations play a role in CRCs, not all mutations present direct neoplastic transformations. Only a few of them regulate neoplastic transformation of CRCs. Among the genes implicated in CRCs, survivin, an anti-apoptotic gene, is highly upregulated and is used as a prognostic tool for treatment outcome of CRCs [25]. Multiple class I HDACs are upregulated in a subset of CRCs with 36.4% being HDAC1, and high HDAC expression has been shown to be associated with reduced patient survival in CRCs [26]. DNA hypermethylation of tumor suppressor genes have also been shown to frequently occur in CRCs [27, 28]. However, DNA hypermethylation and hypomethylation effects are dependent on the gene in question. Hypermethylation of tumor suppressors and hypomethylation of oncogenes induce tumor forming potentials. In CRCs, DNA
methylation and histone modifications are not considered as independent events but rather function through crosstalk catalyzed by distinct enzymes that control gene expression [29]. Dietary compounds with epigenetic properties are considered suitable for the treatment of CRCs as diet influences the health of the normal colon and also regulate the type of microbiome of the colon. A fiber-rich diet promotes butyrate-producing bacteria [30, 31]. These dietary molecules are considered safe and are easily obtained with minimal side effects. We therefore sought to investigate the combined chemotherapeutic effects of EGCG and NaB in CRCs through the promotion or inhibition of epigenetic functions that influence gene expression.
MOLECULAR MECHANISMS FOR INHIBITION OF COLON CANCER CELLS BY COMBINED EPIGENETIC-MODULATING EPIGALLOCATECHIN GALLATE AND SODIUM BUTYRATE.

by

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Abstract

Bioactive compounds are considered safe and have been shown to alter genetic and epigenetic profiles of tumor cells. However, many of these changes have been reported at molecular concentrations higher than physiologically achievable levels. We investigated the role of the combinatorial effects of epigallocatechin gallate (EGCG), a predominant polyphenol in green tea, and sodium butyrate (NaB), a dietary microbial fermentation product of fiber, in the regulation of survivin, which is an overexpressed anti-apoptotic protein in colon cancer cells. For the first time, our study showed that the combination treatment induced apoptosis and cell cycle arrest in RKO, HCT-116 and HT-29 colorectal cancer cells. This was found to be regulated by the decrease in HDAC1, DNMT1, survivin and HDAC activity in all three cell lines. A G2/M arrest was observed for RKO and HCT-116 cells, and G1 arrest for HT-29 colorectal cancer cells for combinatorial treatment. Further experimentation of the molecular mechanisms in RKO colorectal cancer (CRC) cells revealed a p53-dependent induction of p21 and an increase in nuclear factor kappa B (NF-κB)-p65. An increase in double strand breaks as determined by gamma-H2A histone family member X (γ-H2AX) protein levels and induction of histone H3 hyperacetylation was also observed with the combination treatment. Further, we observed a decrease in global CpG methylation. Taken together, these findings suggest that at low and physiologically achievable concentrations, combinatorial EGCG and NaB are effective in promoting apoptosis, inducing cell cycle arrest and DNA-damage in colorectal cancer cells.
Abbreviations: EGCG, epigallocatechin gallate; NaB, sodium butyrate; HDAC, histone deacetylase; HAT, histone acetyltransferase; DMSO, dimethylsulfoxide, DNMT1, DNA methyltransferase 1; DNMT3A, DNA methyltransferase 3A; γ-H2AX, gamma-H2A histone family member X; NF-κB, nuclear factor kappa B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; PI, propidium iodide; PCR, polymerase chain reaction; ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; TBS-T, tris buffered saline-tween 20

Keywords: Epigenetics; Colon cancer; Sodium butyrate; Epigallocatechinese gallate; Methylation; Histone deacetylases

Introduction

Survivin, a member of the inhibitor of apoptosis protein family, plays a bifunctional role, as an anti-apoptotic protein and also as a regulator of cell cycle progression [1,2]. It associates with the mitotic spindle during the cell cycle and serves as a check point for correct association of the spindle with chromosomes in metaphase [2]. In colorectal cancers (CRCs), survivin is overexpressed but its expression in normal adult tissue is undetectable [3]. The expression of survivin has been linked to poor survival, recurrence rate and death due to CRCs [4]. Chemoregulatory expression of this protein is therefore a promising target for cure.

The incidence rate of colon cancer is lower in Asian countries where the diet is predominantly rich in vegetables and fruits [5]. The constituents of these dietary foods provides for a healthy colonic environment. Dietary fiber, an important dietary
constituent, ensures that potential carcinogens are removed from the colon and the microbiota within the colon converts the fiber into short chain fatty acids (SCFA) by the process of fermentation [6]. These short chain fatty acids are a major source of energy for the colon cells. Of the SCFAs, butyrate is the predominant energy providing source [7] and is a natural epigenetic regulator functioning as an inhibitor of histone deacetylases (HDACs) [8]. Sodium butyrate (NaB) can induce cell differentiation, apoptosis and histone hyperacetylation [8–10] and these tumor inhibitory properties of butyrate can be exploited as part of a treatment for CRCs.

Another dietary epigenetic molecule, epigallocatechin gallate (EGCG), is a predominant constituent of green tea polyphenols, and regulates epigenetic changes by altering methylation profiles of genes through its DNA methyltransferase 1 (DNMT1) inhibitory activity [11]. Combination therapies incorporating EGCG with other bioactive molecules may be very effective in numerous cancers [12]. However, many of these studies employ high concentrations of the compound that may not be achievable in vivo. Our rational is that when two effective compounds with potent epigenetic properties are used the combined epigenetic effects may be more effective in reducing survivin expression, an upregulated anti-apoptotic molecule in CRCs, and that this may allow lower concentrations of the compounds for therapy. Studies in various other cancer cell lines have shown that EGCG and NaB can effectively inhibit survivin independently, albeit at higher concentrations [13,14]. However, the combination effects of these compounds on colon cells, where the availability of the molecules are at the highest physiological levels, are not known.
In our study, we treated RKO, HCT-116 and HT-29 CRC cells at physiologically achievable concentrations of EGCG and NaB (10 µM and 5 mM, respectively) [15–18] and the combined effects of these epigenetic regulators were observed in terms of survivin down-regulation. RKO and HCT-116 are colorectal carcinoma cell lines and are genetically similar. HT-29 is not genetically similar to RKO or HCT-116 cell lines and is an adenocarcinoma cell line. We sought to determine if the compounds were effective against cell lines that were genetically similar or different, and if p53 would govern the molecular changes observed in the study. We also assessed p21, an important cell cycle regulatory protein that has been reported to regulate survivin expression in other cancer cell types [19,20]. We asked if the combined therapy of EGCG and NaB could have a greater effect at inducing p21 expression with the concomitant down-regulation of survivin in CRC cells, at lower molecular concentrations. NaB alone is potent enough to induce DNA-damage, and when combined with EGCG this damage may be enhanced, stimulating cell cycle arrest in parallel with p21 induction and down-regulation of survivin. We found that the combination of EGCG and NaB arrested cells in the G2/M phase for both the RKO and HCT-116 CRC cells and a G1 arrest was observed in HT-29 cells. All cells had a decreased S phase. p21 induction was observed in the RKO CRC cell which was p53-dependent. Taken together this study provides a novel chemotherapeutic approach in the treatment of CRCs at lower effective doses of natural molecules.
Materials and methods

Cell culture

RKO (CRL-2577), HCT-116 (CCL-247) and HT-29 (HTB-38) CRC cells were obtained from American Type Culture Collection (ATCC). RKO CRC cells were cultured in DMEM 1X medium (Mediatech Inc., Manassas, VA, USA), HCT-116 and HT-29 were cultured in DMEM-F12 (Mediatech Inc., Manassas, VA, USA), and all cell cultures were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 1% penicillin/streptomycin (Mediatech, Herndon, VA, USA). The cells were cultured as per the manufacturer's protocol and were maintained in a humidified 5% CO₂ incubator at 37°C. RKO, HCT-116 and HT-29 CRC cells were treated with 10 μM EGCG (Sigma, St. Louis, MO, USA) or 5 mM sodium butyrate (NaB) (Sigma, St. Louis, MO, USA) for 48 h. EGCG was prepared in DMSO with a stock concentration of 20 mg/ml and NaB was at a stock concentration of 100 mg/ml in sterile water. The concentration of DMSO in medium was less than 0.1% (v/v). Cells treated with DMSO served as a vehicle control. During treatments working solutions were freshly prepared and the medium was changed every 24 h with the freshly prepared compound solutions.

Cell viability assessment

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after treatment with various concentrations of EGCG and NaB and selected concentration of the combined drugs. Approximately 1×10⁴ RKO, HCT-116 and HT-29 CRC cells were seeded in each well in
96-well plates. Cells were treated as indicated after 24 h. At the end of each treatment the cells were washed twice with 100 μL PBS and 100 μL of media containing 10 μL of 1 mg/mL MTT (Sigma, St. Louis, MO, USA) was added to each well before incubation for 1 h at 37°C in a humidified 5% CO₂ incubator. At the end of the incubation period, the medium was aspirated and 200 μL DMSO was added to each well to dissolve the formazan crystals. Dye absorbance in each well was measured at 490 nm with a reference wavelength at 620 nm. Cells treated only with media served as negative control and DMSO at a final concentration of 0.1% was used as experimental control.

**Analysis of cell cycle progression**

Propidium iodide (PI) staining-based flow cytometry cell cycle assay was used to analyze cell cycle distribution. Approximately 5×10⁴ RKO, HCT-116 and HT-29 CRC cells were plated in each well of 6-well plates with a 2 mL volume of medium for each well. Medium containing freshly dissolved EGCG or NaB was added 24 h later and changed daily. Harvesting of the cells was carried out at the indicated time points from 6-well plates by trypsinization. After washing with PBS, cells were fixed in 70% ethanol at 4°C overnight and centrifuged and washed with PBS the second day. Before analysis, incubation of cells was carried out in the dark for about 30 min in PBS containing 0.1% Triton X-100, 0.1% RNase, and 50 μg/mL PI. DNA contents in stained nuclei were then analyzed with flow cytometry.
Apoptosis analysis

Annexin V and PI double-staining-based flow cytometry apoptosis assay was used to determine the effect of EGCG and NaB treatment on apoptosis. Cells were harvested with trypsinization followed by washing with PBS and staining with Annexin V and PI (Invitrogen) in the binding buffer for 15 min in the dark. Subsequently, the cells were analyzed through flow cytometry.

Protein expression analysis

Western blotting was employed to assess protein expression. After cells were washed with PBS, protein lysates were obtained in RIPA lysis buffer containing protease inhibitors (Upstate Biotechnology, Charlottesville, VA, USA). Ten micrograms of protein sample was electrophoresed on 4–10% Tris glycine SDS-polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes. Nuclear lysates were assessed for p21, p53 and NF-κB-p65. Total protein was used to assess HDAC1, DNMT1, DNMT3A and DNMT3B, survivin, anti-acetylated H3 and γ-H2AX. Total protein analysis instead of acid extraction was performed to determine anti-acetylated H3 and γ-H2AX levels as they have been determined in the similar manner and has been previously reported [21,22]. Antibodies against p21 and anti-acetylated H3 (Millipore, CA, USA), HDAC1, DNMT1, NF-κB-p65 (Abcam), p53, survivin, DNMT3A, DNMT3B (Santacruz Biotechnology) and γ-H2AX (Cell Signaling) were used to probe the corresponding proteins. Briefly, blots were blocked in 5% Milk powder TBS–T solution for 30 min. Primary antibody was added in 1% Milk powder TBS–T solution for 1 h at room temperature with shaking; for survivin and γ-H2AX, incubation with primary antibody
was carried out at 4°C overnight, the rest were performed at room temperature for 1 h. DNMT3A and DNMT3B and survivin were used at 1:100 dilution and the rest were used at 1:1000 dilution. Blots were washed for 30 min at 10 min each wash. Secondary antibody at 1:1000 dilution was added for 1 h at room temperature. Blots were washed with TBS–T for 30 min at 10 min each wash and immunoreactive bands were visualized with the enhanced chemiluminescence detection system (Millipore) using the BIORAD chemidoc XRS image. Proteins were identified based on their molecular weights compared to the standard that was run in parallel and transferred to the blot. The western blots were conducted in triplicates and densitometric analysis of the bands were performed using myImageAnalysis1.1 (Thermo Scientific).

**Real-time quantitative PCR**

Total cellular RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA (100 ng) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-rad, Hercules, CA, USA). The PCR primer sets that were used are the following: 5′-ACCACAGTCCATG CCATCAC-3′ (F), 5′-TCCACCACC CTGTTGCTGTA-3′ (R) for GAPDH; 5′-CACT CCAAAACGCCGGCTGATCTTC-3′ (F), 5′-TGTAGAGC GGGCCCTT TGAGGCCCTC-3′ (R) for p21; 5′-ACCGCTTCTACTTT CCTGAGG CCTA-3′ (F) 5′-GTTGCAGTCC TCTGTGAACACTGTGG-3′ (R) for DNMT1; 5′-GACGGGGATGTT GGAAATTA-3′ (F), 5′-CATCTTC ACATGGGGTG-3′ (R) for HDAC1; 5′-ATGGACGATCTGTTT CCCCT-3′ (F), 5′-CGGTTTACTCGGCAGATCTT-3′ (R) for NF-κB-p65; and 5′-GC CAT GGGTGCCCCGAGGTG-3′ (F), 5′-GCTCCGGCCAGGGCCT CAA-3′(R) for
survivin. GAPDH was used as an internal loading control. Real-time quantitative PCR was conducted in a CFX96 Real-Time PCR System (Biorad) using SYBR Green detection system (Bio-rad, Hercules, CA, USA). The delta–delta CT method was used to determine the relative level of gene expression. Fold change in gene expression was determined by the formula $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T=\Delta C_T(\text{target})_{\text{normalized}}-\Delta C_T(\text{Control})_{\text{normalized}}$; $\Delta C_T(\text{target})_{\text{normalized}}=C_T\text{target (gene of interest)}-C_T\text{ target (GADPH)}$ and $\Delta C_T(\text{Control})_{\text{normalized}}=C_T\text{control (gene of interest)}-C_T\text{ control (GADPH)}$.

### Colonogenic assays

The cells were seeded at a density of 100 cells per well in a 24-well plate and allowed to recover for a day prior to the experiment. EGCG and NaB were added to the wells at the appropriate concentrations. After the 48 h time period, the media was removed and fresh media was added and the plates were incubated at 37°C at 5% CO₂ for a week. The wells were washed with 1X PBS and the colonies were fixed using glacial acetic acid and methanol at the ratio 1:7 for 5 min. Crystal violet (0.5%) in PBS was added and kept for 30 min. The wells were gently washed and allowed to air dry. Colonogenic assays were performed in two ways: (1) Cells were first treated to the compounds and then colony formation potential was assessed and (2) the plated cells were allowed to form colonies prior to treatment and then exposed to treatment to determine colony reduction potential. Percent values were obtained by comparing to control.
HDAC activity and CpG percent methylation

The epigenetic changes were assessed using the Epigenase HDAC and Methylflash methylated DNA Epigentek kits as per the manufacturer’s protocol. For HDAC assays 20 µg/µl of nuclear protein was used and for percent methylation 100 ng of genomic DNA was used. The absorbance was measured at 450 nm using the Bio rad 680 plate reader. Activities for each were calculated based on manufacturer’s instructions.

Chromatin immunoprecipitation (ChIP) assay

RKO CRC cells were seeded in a 10 cm dish and were subjected to the respective treatments for 48 h. After treatment the cells were fixed with 37% formaldehyde solution and then subjected to enzymatic shearing as per the manufacturer’s protocol (ChIP-IT express enzymatic kit, Active Motif). p53 antibody (3 µg, mouse monoclonal, ab26 Abcam) was used to pull down the DNA protein complex. After reversing the crosslinks, the p53-enriched DNA and input DNA was purified and then subjected to PCR amplification using primers for p21 promoter sequences that amplify the region near the p53-binding site: 5’-GCACTCTTGTCGCCAG-3’ and 3’-TCTATGCCAGAGCTC AACAT-5’. The amplified product was electrophoresed on a 3% agarose gel and the density of the bands was measured using the myImageAnalysis software (ThermoScientific). The density of p53-enriched samples was normalized to the input sample.
**Statistical analyses**

Statistical significance among treatments was evaluated using one-way ANOVA followed by Tukey test. *$P<0.05$ and **$P<0.01$ were considered significant.

**Results**

**EGCG and NaB suppresses RKO CRC cell proliferation**

As an individual compound, EGCG was less effective than NaB at inhibiting the cell proliferation of RKO CRC cells (10% at 10 μM) at lower concentrations (Fig. 1A). The IC$_{50}$ for EGCG was about 50 μM. However, the bioavailability of EGCG is relatively poor at 0.1 μM plasma levels although it is delivered at 1–20 μM in the colon based on the dose of green tea consumed [23,24]. NaB was more effective at inhibiting the cells. At the 5 mM NaB dose, which is considered achievable in the colon [25], about 35% of the cells were inhibited. It is notable that the combined effect of 10 μM EGCG and 5 mM NaB resulted in 50% cellular inhibition suggestive of an additive effect at this IC$_{50}$ value for the combined treatment. The additive effect of the combined treatment on HCT-116 and HT-29 CRC cells was significant at higher IC values than at IC$_{50}$. For consistency and given that at these doses had an additive effect, the same concentrations were used for HCT-116 and HT-29 CRC cells (Fig. 1B). All three cell lines chosen are positive for the CpG island methylator phenotype, indicative of a higher degree of genomic methylation in these cells [26]. In addition, RKO and HCT-116 are p53 wild-type (wt) and are colorectal carcinoma cell type. HT-29 cells carry a mutation in the codon 273 in p53 and are adenocarcinoma cell types [26]. We wanted to test the applicability and effectiveness of the combination in cell lines having similar and
Fig. 1 EGCG and NaB inhibits the proliferation of RKO, HCT-116 and HT-29 CRC cells. The effective dose of the combination for EGCG and NaB was determined at IC$_{50}$ for the combined effect (A). EGCG and NaB independently inhibit the proliferation of RKO CRC cells, with NaB having a greater effect at physiological concentrations. The combination effect of EGCG and NaB at physiological available doses (10 μM and 5 mM EGCG and NaB, respectively) produced a significant additive effect in terms of inhibition of cell proliferation in RKO CRC cells (A). Significant additive effects were also observed for HCT-116 and HT-29 CRC cells at the same doses, albeit at higher IC values for the combination (B). Morphology of RKO, HCT-116 and HT-29 CRC cells after various treatments is shown at 100× magnification (C). *$P$<0.05, **$P$<0.01
different genetic characteristics. Morphological changes associated with the respective treatments were observed at 100× using a Leica DM 750 phase contrast microscope (Fig. 1C). As compared to non-treated control, the RKO CRC cells in the combination appeared to gain a more spindle, flattened and circular characteristic. However, with EGCG alone a very slight morphological change was observed which was consistent with the efficacy to the cancer cells at the concentration of 10 μM. Prominent morphological changes were visible with 5 mM NaB. The combination of EGCG and NaB resulted in pronounced changes of a larger number of circular shaped cells from the normal epithelial-like morphology, indicative of the enhanced effect of EGCG with NaB (Fig. 1C). More pronounced morphological changes were also observed for HCT-116 and HT-29 CRC cells with NaB treatment only and the combination treatment as compared to the control and EGCG treatment only.

**EGCG and NaB promotes apoptosis**

Induction of apoptosis is one of the major pathways through which chemotherapeutic agents inhibit tumor proliferation. We therefore examined the apoptotic potential of the combination treatment of RKO, HCT-116 and HT-29 CRC cells. We found that the combination of 10 μM EGCG and 5 mM NaB induced significant apoptosis ($P<0.01$) in comparison to the individual treatments at 48 h (Fig. 2). However, the apoptotic induction was not high enough to be considered the sole mechanism of inhibition of cell proliferation. We therefore believe that a co-mechanism in addition to apoptosis may be involved in the observed inhibition of cell proliferation.
**Fig. 2** EGCG and NaB combination can induce apoptosis of CRC cells. Cells were treated with 10 µM EGCG and 5 mM NaB and after a 48 h time period the percent apoptotic cells were analyzed by flow cytometry. Apoptosis cells include both early apoptosis and late apoptosis. A significant increase in the percent apoptotic cells was observed for the combination treatment as compared to the individual treatments. (*P<0.05, **P<0.01). The experiment was repeated three times.
EGCG and NaB arrest RKO, HCT-116 CRC cells predominantly in the G2/M phase and HT-29 CRC cells in the G1 phase

Cell cycle progression analysis revealed a shift from S to the G2/M phase (Fig. 3). The cells were predominantly in the S phase for EGCG at 10 μM concentration. However, with NaB treatment at 5 mM a G2/M arrest was observed for RKO CRC cells and in the combination treatment the cells were arrested in the G1 and G2/M phases. In HCT-116 cells a significant G2/M arrest was observed and in HT-29 cells a significant G1 arrest was observed \( (P<0.01) \) as compared to the controls (Fig. 3). In comparing all three cell lines a reduction in S-phase was observed indicative of a possible mechanism of preventing cell proliferation that may be associated with DNA damage.

**EGCG and NaB effectively inhibit colony formation in RKO CRC cells**

From our apoptotic and cell cycle arrest results we next sought to determine if the combination was effective in inhibiting the colony forming potential of RKO CRC cells. Our results indicate that the combination was significantly more potent in terms of reducing the number of colonies than individual treatments. Both types of colonogenic assays showed that the combination was successful in inhibiting/reducing RKO cellular tumor forming potential (Fig. 4A, B and C). The combination significantly inhibited colony formation by 80%, while EGCG and NaB administered singly were about 20% and 50% effective, respectively. These changes were found to be significant at \( P<0.01 \).

Thus, EGCG significantly enhances the effect of NaB in reducing colony formation. Although slightly different efficacies were observed with respect to Fig. 4A, the reverse colonogenic assay showed similar trends in colony reduction potential. EGCG reduced the number of colonies formed by 10%, NaB by 40% and the combination by 70%.
Fig. 3 EGCG and NaB in combination can induce cell cycle arrest in CRC cells. A shift towards the G2/M phase was observed in the combination treatment (10 μM EGCG and 5 mM NaB) as compared to controls for both RKO and HCT-116 CRC cells and the change was significant. A significant G1 arrest was observed for HT-29 CRC cells. Therefore, depending on the cell line under study the combination treatment was able to arrest cells in G1 and G2/M phases. *P<0.05, **P<0.01. The experiment was repeated two times and each point indicates the mean±SEM.
Colony formation potential of RKO CRC cells. Colony forming potential of RKO CRC cells was assessed by treating the cells with 10 µM EGCG and 5 mM NaB for a 48 h time period and colonies formed in the absence of the compounds after a week of incubation were counted. Values are represented as percent control ± SEM of two independent experiments performed in triplicates. Representative photographs are shown from the experiments. (A). Colonies that had greater than 50 cells were counted. A significant decrease in colony formation was observed for the combination treatment as compared to the individual treatments. Morphology and sizes of the colonies formed are shown in (B). Reverse colonogenic assay was also performed to determine compound effectiveness in reducing colonies once formed (C).
changes were significant with respect to the control ($P<0.01$). Also, the combination was significantly different from the individual treatments alone ($P<0.01$). These data indicate that the combination is potent in inhibiting as well as reducing colonies formed, activities that are important to effective chemotherapeutic strategies.

**EGCG and NaB increases $p21$, $NF-\kappa B$-$p65$, $HDAC1$; and decreases $DNMT1$ and survivin in RKO CRC cells**

We examined the effect of the combination on DNMT1 and HDAC1 levels and found that DNMT1 levels decreased for the combination treatment, whereas HDAC1 levels increased (Fig. 5A). Findings from other studies point to the existence of a regulatory feedback mechanism that modulates HDAC levels, which was also observed in this study [27]. Real time PCR analysis of RKO CRC cells revealed that $p21$ levels increased for the individual treatments and was highly induced with the combination treatment. EGCG was effective in enhancing NaB induction of $p21$ (Fig. 5B). We also determined the effect of the combination treatment on survivin expression as it is transcriptionally regulated by $p21$. Survivin mRNA levels decreased (Fig. 5B) in response to the combination treatment and was significant ($P<0.01$) as compared to the control. HDAC inhibitors are known to transactivate $NF-\kappa B$ and NF-κB is associated with $p21$ dependent G2/M arrest. We therefore determined the effect of the treatments on $NF-\kappa B$-$p65$. Ours is the first study to report that $NF-\kappa B$-$p65$ levels were significantly up-regulated for NaB alone and for the combination treatments of EGCG and NaB (Fig. 5C). However, the combination was lower than NaB treatment but was significantly higher than EGCG alone ($P<0.01$).
Fig. 5 Effects of EGCG and NaB on the mRNA expression of specific epigenetic, cell cycle and cell proliferation modulators in RKO CRC cells. RKO CRC cells were treated to 10 μM EGCG and 5 mM NaB and after a 48 h time period RNA was extracted. Real time assessment of mRNA expression of the various genes revealed that DNMT1 and survivin expression decreased significantly for the combination treatment as compared to EGCG and control (A, B). EGCG is a known DNMT1 inhibitor although its effects are more pronounced at the protein than mRNA level. However, HDAC1 and p21 (A, B) increased several fold and the increase was significant when compared to the control and individual treatments. A significant increase in NF-κB-p65 was observed for both the NaB alone and the combination treatment (C). However, the combination treatment was lower as compared to the NaB alone treatment, and significant and relatively higher when compared to EGCG and the control. Data are in triplicates from three independent experiments. *P<0.05, **P<0.01.
EGCG and NaB inhibits HDAC1, DNMT1 and survivin in all the three CRC cells tested

EGCG and NaB are epigenetic modulators and therefore we assessed the levels of DNMT1 and HDAC1 for the individual treatments and the combination treatment in all three cell lines. Epigenetic proteins HDAC1 and DNMT1 levels were significantly reduced in all three cell lines for the combination indicative of a combinatorial epigenetic effect of the compounds under question (Fig. 6A and B, respectively). EGCG directly inhibits the catalytic site of DNMT1 via its gallic acid moiety [11]. Sodium butyrate has
Fig. 6 Effect of the combinatorial compounds on the expression of HDAC1, DNMT1 and survivin in RKO, HCT-116 and HT-29 CRC cells. The combination treatment was significantly effective in down-regulating the expression of HDAC1, DNMT1 and survivin in all the three CRC cells tested (A, B, C, respectively). The western blot was performed in triplicate and densitometric analysis of the bands was performed. The data represent values normalized to β-actin and then computed as relative to the control. A representative western is shown as an inset in the graph for each protein type tested. C=control; E=EGCG; N=NaB and E+N=EGCG+NaB. Survivin is highly expressed in CRCs and we therefore determined the effect of the combination in regulating survivin expression. Survivin expression was significantly inhibited with the combination treatment as compared to the control ($P<0.01$) in all three cell lines (Fig. 6C). In RKO CRC cells the changes in survivin expression were significant in the combination as compared to the individual treatments (Fig. 6C).
been shown to decrease DNMT1 levels in breast and prostate cancer cells via proteasomal degradation and may be a possible mechanism of the inhibition seen in our study [28]. The combination of EGCG and NaB in significantly reducing DNMT1 levels may be due to the combination of inhibiting the catalytic site along with proteasomal degradation.

**EGCG and NaB activates nuclear p53, p21, NF-κB-p65; activates γ-H2AX and anti-acetylated H3; inhibits DNMT3A and DNMT3B in total protein assessed in RKO CRC cells**

Densitometric analysis of p53, p21, and NF-κB-p65 from nuclear protein revealed an upregulation of the proteins in the combination treatment and was significant in comparison to the control ($P<0.01$) (Fig. 7A). NF-κB-p65 induction may be linked to DNA damage and therefore we assessed the levels of γ-H2AX, the expression of which indicates DNA damage. Our results showed an induction of the protein which was significant as compared to the control ($P<0.05$) (Fig. 7B). Sodium butyrate is a HDAC inhibitor and therefore we assessed the acetylation status of histone H3 and found a significant increase in the combination treatment as compared to the control. A significant ($P<0.05$) decrease in DNMT3A and DNMT3B levels was also observed in the combination treatment as compared to the controls (Fig. 7C).
Fig. 7 Effect of the combinatorial compounds on the expression of p21, p53 and NF-κB-p65 from nuclear lysates; γ-H2AX, anti-acetylated H3, DNMT3A and DNMT3B from total protein lysates, in RKO CRC cells. Nuclear proteins were analyzed in triplicates for p21, p53, and NF-κB-p65 in RKO CRC cells (Fig. 7A) and total protein in triplicates was assessed for γ-H2AX and anti-acetylated H3 levels (Fig. 7B). At 10 μM EGCG and 5 mM NaB combination, p21 levels increased in RKO CRC cells. An induction in p53 expression was also observed in the combination treatment. NF-κB-p65 levels increased in the combination and NaB treatment when compared to the control and EGCG treatment. This increase may be associated with the double-strand breaks (DSBs) indicated by increased γ-H2AX levels (7B) and G2/M arrest as was found in the study. De novo methylation-associated epigenetic proteins DNMT3A and DNMT3B levels also decreased for the combination and this was tested only in RKO colon cancer cells (Fig. 7C). An increase in anti-acetylated H3 levels was also observed with the combination (Fig. 7B), an epigenetic change probably associated by the decrease in HDAC1 levels and HDAC activity. *P<0.05, **P<0.01.
EGCG and NaB induces p21 through a p53-dependent mechanism in RKO CRC cancer.

Induction of both p21 mRNA and protein were observed in RKO CRC cells. To determine if the induction of p21 was p53-dependent we performed the ChIP assay using a monoclonal p53 antibody. ChIP-PCR analysis using primers specific to the p21 promoter region showed a significant increase in p21 levels for the combination (Fig. 8). These results suggest a p53-dependent mechanism of p21 induction in RKO CRC cells.

**Fig. 8** p53-dependent induction of p21 by EGCG and NaB combination treatment in RKO CRC cells. ChIP-PCR analysis of RKO CRC cell p53-enriched chromatin showed a significant upregulation of p21 in the combination treatment. Primers chosen were specific for the p21 promoter region. The increase in p53-enriched p21 levels in the combination is indicative of a p53-dependent mechanism of p21 induction in RKO CRC cells. The result is in agreement with the increase in mRNA and protein levels of p21 observed in the study. PCRs are from a single experiment repeated three times *P<0.05, **P<0.01
EGCG and NaB affect global DNA methylation and chromatin structure

Epigenetic mechanisms control gene expression through changes predominantly in DNA methylation and histone acetylation. The EGCG and NaB compounds that we used in the study are potent DNMT1 and HDAC1 inhibitors, respectively, and therefore, the epigenetic effects in the presence of two different types of epigenetic inhibitors were assessed. HDAC activity was determined for all three cell lines and the activity significantly decreased in the combination treatment as compared to the control for all of the three cell lines \((P<0.01)\) (Fig. 9A). No significant changes in HAT activity was observed in the combined drug treatments (data not shown). Percent CpG methylation was assessed only for the RKO cell line as the level of three DNMT proteins were determined through western blots for this cell line only. Percent CpG methylation significantly decreased for the combination treatment of EGCG and NaB for RKO cells \((P<0.01)\) (Fig. 9B). The increase in percent methylation for the NaB treatment may be attributed to an increase in DNMT3B levels as was observed with the protein densitometric analysis. The treatment of the cells that alter dominant epigenetic enzymes may account for the observed changes in percent methylation and HDAC activity.
Fig. 9 Effect of EGCG and NaB on HDAC activity in all the three CRC cells and percent CpG methylation in RKO CRC cells. The combination treatment (10 µM EGCG and 5 mM NaB) was significantly effective in decreasing HDAC activity (*P<0.05, **P<0.01) in all three cell lines tested (A). Percent CpG methylation was analyzed in RKO CRC cells and the decrease observed in the combination treatment was significant as compared to the individual treatment (B). The increase in percent methylation of CpGs with NaB may be due to the compensatory effects of de novo methylating enzymes such as DNMT3B. Data are from three independent experiments and represent mean±SEM.
Discussion

Sodium butyrate (NaB) is non-toxic and is produced naturally within the colon through microbial fermentation. The concentrations of NaB are effective in the millimolar range and 1 mM is considered low, 5 mM intermediate and 10 mM high [29]. We considered the intermediate concentration in terms of treatment as it was effective with 10 μM EGCG in reaching an IC\textsubscript{50} value for the combination within 48 h of treatment for RKO CRC cells and these concentrations are achievable \textit{in vivo}. NaB has shown promise as a suitable chemotherapeutic agent due to its myriad anti-tumorigenic effects, including cell proliferation inhibition, induction of cell cycle arrest and differentiation, promotion of apoptosis, and even more significantly as a potent HDAC inhibitor inducing histone hyperacetylation and altering gene expression [6,8,30,31]. Sodium butyrate has been shown to induce p21 expression that is a prominent cell cycle regulatory protein [32,33]

In the current study we showed that the potent anti-cancer activities of EGCG and NaB in RKO, HCT-116 and HT-29 CRC cells. On the contrary, a study showed that EGCG interferes with butyrate-induced differentiation in CRC cells by preventing the cellular uptake of NaB [34]. However, in the study conducted, the dose chosen was based on the optimal values of the individual compounds and not on the combination of the compounds. Two given compounds can act differently at different dose combinations. From our MTT assays we showed that at 2 mM NaB and 10 μM EGCG there was no additive or synergistic effect observed in HT-29 CRC cells which was the same cell line chosen for the previous study [34]. Therefore, dose appears to be crucial in determining chemotherapeutic effects. Based on the dose chosen we observed significant changes in morphology, apoptosis, cell cycle arrest and epigenetic proteins for the HT-29 CRC cell
line. In addition, survivin, an antiapoptotic protein highly expressed in colon cancers, was down-regulated in the HT-29 CRC cell line with the combination as compared to EGCG alone. RKO and HCT-116 CRC cell lines also showed significant morphological changes after the combination treatment. Epigenetically and genetically RKO and HCT-116 CRC cells are more similar than HT-29. The presence of wt-p53 in RKO and HCT-116 CRC cells may produce an improved response to the combination treatment than HT-29 CRC cells that carry mutated p53. Reinduction of p53 perhaps may be important in encouraging apoptosis or cell-replicative inhibition which may be p53-dependent. The cell lines chosen were also CIMP positive, which is described in detail elsewhere [26].

Most of our analysis was performed in detail in the RKO wt-p53 CRC cell line and where comparisons were necessary, we included the other cell lines as well.

From our in vitro colonogenic assays we were able to show that a combination of EGCG and NaB was significantly effective in inhibiting colony formation in RKO CRC cells by 80%, when many studies show that either EGCG or NaB acting alone require higher concentrations and time periods for the same effective inhibition. Clearly, the combination treatment at lower physiologically relevant doses is able to generate a significant chemotherapeutic effect suggestive of strong activity against colorectal cancer. Our study also showed that the combination treatment of EGCG and NaB was successful in inducing apoptosis in all three cell lines we treated but was in the range of 12–16% and therefore the apoptotic pathway may not be the only mode of tumor-inhibition by the compounds we evaluated.

In addition to the induction of apoptosis, we found that the EGCG and NaB combination induced cell cycle arrest predominantly in the G2/M phase for RKO and
HCT-116 CRC cells and G1 phase for HT-29 CRC cells. NaB has been shown to exert cell cycle inhibitory effects in the G1 phase or G2/M phase based on the cell line under study [35]. In addition, down-regulation of survivin via p21 induction and p53 upregulation provides an alternative strategy of cell replicative inhibition through DNA damage. Our study for the first time showed that the combination therapy was successful in down-regulating the expression of survivin both at the mRNA and protein levels with p21 induction and G1 and G2/M arrests. We believe that this novel mechanism involved in the inhibition of CRC cells could be applicable to tumors that are resistant to apoptosis.

Genetic and epigenetic changes are instrumental in promoting neoplastic transformation in CRCs [36]. The reversibility of these changes presents a suitable treatment strategy for colorectal cancers. Previous studies have shown that EGCC and NaB are potent DNMT1 and HDAC1 inhibitors, respectively, both of which can individually correct epigenetic observations in cancer cells [8,11]. In our study, NaB was used and is a known HDAC1 inhibitor, and is the most extensively studied HDAC. Contradictory to the norm of gene and protein regulation, our study showed that the HDAC1 mRNA levels were significantly upregulated with the down-regulation of HDAC1 level for the NaB treatment alone and the combination treatment of EGCG and NaB. The upregulation of HDAC1 and the concomitant decrease in protein level may be attributed to the autoregulation of HDAC1 by HDAC inhibitors and could be cell-type specific [37]. HDAC1 has been found to be recruited to its own promoter thereby mediating its own repression by negative feedback loop inhibition [37]. In addition, HDACs are known to undergo several post-translational modifications, of which acetylation, phosphorylation, sumoylation and ubiquitination have been reported to occur.
with HDAC1 that can affect their activity and stability [38]. Taken together, the binding of NaB to the catalytic site of HDAC1 and possible post-translational effects on the protein may account for the decrease in HDAC1 levels despite the increase in HDAC1.

DNA methyltransferases and histone enzymes are no longer considered independent epigenetic regulators but are thought to function in tandem or in cohesion to alter the epigenome. Global CpG methylation response to NaB treatment has been shown to vary on the basis of the cell-type under study [39]. HeLa cells treated with 3 mM NaB for 48 h have shown an increase in global genomic methylation as compared to the control and in glioblastoma multiforme cells a hypomethylated state has been observed at 2 mM NaB for 48 h [40]. The findings from our results showed that percent CpG methylation for NaB increased in comparison to the control. A similar finding has been observed in transformed lung fibroblast cell lines in the presence of 5 mM NaB [39]. At this point we cannot determine the actual reasons for this observation but can infer from the data that the compensatory effects by de novo enzymes may account for this.

A significant decrease in HDAC activity was observed that likely explains the increase in global histone H3 acetylation levels in the combination treatment in comparison to the individual compounds. Histone hyperacetylation has been associated with inhibiting cell growth [41] and therefore we believe that the growth inhibition observed in RKO CRC cells may be due in part to this epigenetic change. Our study showed that in addition to its HDAC inhibitory activity, NaB was able to down-regulate DNMT1 expression as well and the expression further decreased with the combination treatment. Data from other studies support decreased DNMT1 expression, at both the protein and mRNA levels, after HDAC inhibitor treatment [28,42].
The common expressed biomarker signaling DNA damage is $\gamma$-H2AX. Class I, II, and III HDACs have been implicated in the DNA damage response, homologous recombination (HR), and chromatin integrity [43]. HDAC1 have been shown to assist with the DNA damage response by recruiting DNA repair proteins to the site of damage. Butyrate and trichostatin A (TSA) have been show to exert defects in the repair process. Studies with cells lacking HDAC1 have been shown to be hypersensitive to DNA damaging agents and exhibit sustaining DNA damaging signaling reflective of defects in double-strand break (DSB) repair [44]. The results from our study showed a significant decrease in HDAC1 levels in the combination treatment for all three cell lines. Further, an assessment of $\gamma$-H2AX levels in RKO CRC cell lines showed a significant increase in the combination treatment significantly as compared to the control and individual treatments. The increase in $\gamma$-H2AX levels with the concomitant decrease in HDAC1 levels may account for increased DNA damage with decreased cell-replicative capacity. Colonogenic assays performed in RKO CRC cells showed that the combination significantly reduced colony formation and a growth reduction potential was also observed. These findings support the hypothesis that the combination treatment induces DNA damage, arrests the cells in the cell cycle and prevents an increase in cell numbers (data not shown).

HDAC1 has been shown to repress the transcriptional activity of NF-κB-p65. Treatment of the RKO CRC cells to HDAC1 inhibitor NaB in our study increased NF-κB-p65 mRNA and protein, and the expression was higher with NaB treatment and in the combination treatment as compared to the control. Upregulation of NF-κB has been observed in response to DNA damage. HDAC1 has been reported to directly interact with
the p65-subunit of NF-κB and affect its transactivation functions [45]. The inhibition of HDAC1 by NaB in our studies may provide an explanation for the observed increase in nuclear NF-κB-p65 levels where the protein is in its activated state and is associated with DNA damage. Studies have reported that the increase in NF-κB in response to DNA damage may allow for repair of the damaged DNA [46]; however, the repair process is mediated through a host of various other proteins and includes HDAC1. We propose that HDAC1 is a corepressor of NF-κB-p65 and that HDAC1 can directly associate with the p65 subunit of NF-κB. Despite the increase in NF-κB-p65 levels, the decreased levels of HDAC1 may hinder repair functions arresting cells in the cell cycle at the G2/M phase through the induction of p21 as was specifically observed in RKO CRC cells. Thus, EGCG and NaB through their epigenetic modulating effects, in addition to their potent roles as regulators of chromatin structure, and through direct or indirect corepressor or coactivator functions can mediate cellular responses by upregulating genes necessary for cell cycle arrest favoring a therapeutic outcome.

Conclusions

In summary, we have shown that EGCG and NaB with different predominant epigenetic functions are a potent combination with in vitro anti-colorectal cancer properties by suppressing cell proliferation, inducing cell cycle arrest and promoting apoptosis. These results can form the basis of suitable clinical therapies for CRC.

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References


ALTERATIONS IN HISTONE ACETYLA TION IN TUMORIGENESIS

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7.1 INTRODUCTION

Cellular functions, including but not limiting to the production of cell-specific biomolecules, DNA replication and repair, apoptosis, and senescence are intricately and inherently orchestrated by chromosomes [1]. These functions are specified by coding messages in genes. The genetic code, however, is susceptible to alterations, predominantly irreversible mutations, which can result in diseased states at the organismal level. Epigenetic changes involve heritable alterations in gene expression without mutations in the genetic code [2]. Such an outcome is possible as chromatin, the structural component of chromosomes, can undergo changes affecting the condensation states of the chromosome, which allow for or inhibit gene expression [3] (Figure 7.1). These processes are catalyzed by reversible enzymatic modifications. The epigenetics of gene control has gained considerable support and has since been a chosen target for therapeutic intervention for several diseases, in particular cancer.

7.2 NUCLEOSOMES

At the molecular level, the nucleosome constitutes the core of chromatin and consists of basic proteins called histones [2,4]. The 146 bp of DNA complexes with the nucleosome, an octamer, and does so via its negatively charged phosphate backbone. In the octamer, the dimeric H3 and H4 subunits interact in a tetramer formation, whereas H2A and H2B histones remain as dimers [2,4]. The complex does not include the histone protein H1, but this protein, a linker, appears to facilitate the condensation of the nucleosome units
FIGURE 7.1 Histone mediated gene expression. Histone-mediated chromatin fluxes are depicted in the figure. These changes in histone dynamics are brought about by reversible enzymatic reactions mediated by histone acetyltransferases and histone deacetylases. Addition of an acetyl group changes the charge environment surrounding the DNA, as a result the DNA is freed from the nucleosome core, allowing for the transcriptional complexes to bind and induce transcription. Histone deacetylases on the other hand have apposing effects as the neutral charge induced by the acetyl group is removed, allowing the DNA to complex back with the histones, inhibiting expression and other transactivating or repressible elements to the site. Within the same histone subunit, differential alterations can have colossal effects on gene expression under its control. For example, methylation and acetylation of the histone H3 lysine 9 (H3K9) subunit can have apposing effects on gene expression in conjunction with other histone modifications.
lysine residues are modified, in some cases serine or arginine residues are also altered [8–10].

[2,3]. The amino terminal domains of histones protrude out of the complex and are susceptible to enzymatic modification at lysine residues [5–8]. Although predominantly the altered states largely depend on the enzyme catalyzing the modification and the availability of the residues to the enzymes. The type and position of the modification on the histone dictates a pattern, termed the histone code, which specifies the transcriptional regulation of the complexed DNA [11]. This regulation is based upon the availability and accessibility of transcription factors.

7.3 EFFECTS OF NUCLEOSOMAL MODIFICATIONS

There are many modifications known to posttranslationally alter the residues, of which acetylation is the most extensively studied [7,8]. The orientation and modulations of the histones affects the fluidity of the chromatin, resulting in heterochromatin or euchromatin states. In normal cells, the cell cycle is carefully regulated by cell cycle regulatory genes. However, alterations in genes that control tumor suppressors or the cell cycle promote the tumor phenotype. Therefore in abnormal cell growth and division there are three possible outcomes (Figure 7.2): (1) Genes that control cell cycle division are turned off, (2) tumor suppressor genes are turned off, or (3) oncogenes are switched on. This can arise by an imbalance in the acetylation/deacetylation states of the histone residues in the
FIGURE 7.2 Histone acetylation toward a neoplastic phenotype. Involvement of the histone acetylation/deacetylation balance in tumorigenesis. In cells that are normal, genes involved in the cell cycle regulation are expressed and to a certain extent are controlled by histone acetylation of the histones associated with the promoters. [] represents p21 and the [■] represents p53; both are tumor suppressors. However, in response to local hypoacetylation and in conjunction with defects in other cellular pathways, the tumor suppressors are turned off and tumor formation may be induced as the cell cycle is deregulated. In some instances hyperacetylation of oncogenes [△], as in the case with HPVE6, occur that can induce a cancer phenotype.
nucleosome, in addition to other factors. This probably explains why most genes crucial to maintaining the cell cycle balance are turned off or deregulated in neoplasms.

### 7.4 HISTONE ACETYLATION

Histone acetylases, commonly referred to as HATs, recognize the basic substrates and add an acetyl group [8]. The addition of an acetyl group to the lysine residue neutralizes the charge, which relaxes the bound DNA from the histone complex. However, expression of the genes is dependent on the availability of transactivating factors and proteins to the freed up DNA [8]. Moreover, it is important which lysine residues are acetylated because at any given time the position of the lysine and the histone subunit involved in the acetylation largely dictates the downstream effects [8].

Histone molecules are maintained in a delicate balance between acetylated and deacetylated states and work in perfect synchronization for optimal gene function. However, deregulation of this posttranslational balance can essentially lead to the upregulation of oncogenes that enable cells to acquire tumorigenic, metastatic, and invasive phenotypes. Re-acetylation probably prevents such an outcome, as genes crucial to cell cycle regulatory functions are upregulated. Attenuation of deacetylation, with an increase in acetylation of histones, has positive consequences in terms of inhibiting neoplastic growth. Increased acetylation tends to slow down cell growth and induce differentiation and apoptosis, as evidenced by a number of studies utilizing compounds that inhibit histone deacetylases in cancer cell lines. The effect is mediated by the upregulation of proteins like p53 by acetylation of their lysine residues [12,13].
7.5  HISTONE ACETYLATION AND CANCER

HATs exhibit substrate preference toward the lysine moieties that they acetylate. For instance, GCN4, a histone acetylase, preferentially acetylates H4 lysine residues at positions 5, 8, 12, and 16 [14,15]. Acetylation of each of these residues has a different effect. Acetylation of H4 K5 and H4 K12 are merely associated with directing the histones to newly formed DNA in the S phase [14,15]. However, H4 K16 enrichment is involved with actively transcribing genes [14,15]. Thus physiological conditions and requirements of the cell most likely dictate the histone marks. Similarly in histone H3, acetylation by GCN5 at positions 9, 14, 16, and 23 is observed. As in the case of H4, in H3 the function of histone deposition to newly synthesized chromatin is mediated by K9 acetylation [14,15]. The remaining acetylated positions are involved with gene expression [14,15]. Therefore mutations in the enzyme that dictate this function can cause mistargeting of acetylation, changes in the acetylation pattern, and deregulation of acetyl enrichment. These events can disrupt the cell cycle balance and create an environment conducive to the development of cellular transformations.

Acetylation events do not act alone in mediating the downstream effects but require the collaborative effort of other pathways, influenced by other histone codes. In some cases the effect of acetylation at a given residue can rescue the function or provide for redundancy for mutated lysine residues. This phenomenon was clearly demonstrated in a study where the substrates of GCN5 were mutated to determine the overall effects on cell growth. Mutations in all the K residues of H4 resulted in accumulation of cells predominantly in the G2 phase [14]. The observed mutated phenotype was rescued when a lysine residue other than the ones mutated were acetylated, providing for the
redundancy of function. This clearly indicates that acetylation is required for cell viability. Many neoplastic transformations arise from deregulation in genes that affect particular target genes or several pathways. Some of the genes come under direct epigenetic modifications whereas others are regulated indirectly. We have discussed some of the cancers that have arisen due to changes in histone modifications and the target genes they affect.

7.6 INVOLVEMENT OF HISTONE ACETYLATION IN BREAST CANCER

Aberrant histone acetylation can be one of the mechanisms of many different events that result in cancer of breast tissue. Estradiol, an estrogen steroid, binds to estrogen receptors. This complex associates with the estrogen response element and mediates its downstream effects [16]. Interestingly this association also encourages the binding of protein complexes that possess HAT activity. Breast cancers have been distinguished into two categories, estrogen-dependent and estrogen-independent type [16]. In the estrogen-dependent form, the breast tissue is constantly bathed with the steroid molecule, in part due to the ovulation cycle. However, other mechanisms may also promote estrogen synthesis. Over-expression of the hormone can induce changes in the histone acetylation pattern although there is some debate regarding the type of acetylation patterning that occurs. Some studies indicate that an increase in histone acetylation of H4 by estradiol in breast cancer cells occurs [16] while other studies show a decrease in acetylation [16]. Acetylation of lysine residues can achieve different isoforms—mono, di, tri, and tetra. Therefore, in theory, if the lysine is predominantly mono-acetylated, then histones can be relatively easily deacetylated. However, the higher acetylated isoforms can decrease the
rate at which deacetylation occurs. Therefore, the increase in acetylation found in the studies done on breast cancer by estradiol could be mediated by slower deacetylation rates with no change in acetylation rates of higher acetylated isoforms.

All histone subunits are not acetylated/deacetylated at the same rates. Some populations are acetylated faster than the others. Tetra-acetylation of H4 is mediated by rapid acetylation whereas some other histones achieve higher acetylated isoforms much more slowly. A study showed that 60\%–70\% of histones are acetylated in breast cancer [16]. These histones are classified into three categories (1) those that are always acetylated and remain in that state, (2) those that are acetylated/deacetylated quickly, and (3) those that are acetylated/deacetylated relatively slow [16]. The majority of histones fall into the last category (3). However, 10\% of histones belong to category (2) [16]. In addition to acetylated states, if the majority of the isoforms found are of the higher order, then deacetylation of such molecules will be slowed down, culminating in continuous gene expression.

The category (2) histones are the most affected by estradiol. Estradiol increases the rate of histone deacetylation but does not alter the rate of histone acetylation. Hyperacetylated histones H3 and H4 are found to be associated with coding regions of E2-ER (required for HAT activity) and cMyc genes (expressed in proliferating cells) [31]. cMyc is a well-studied oncogene and the upregulation of myc translates into tumor phenotypes. Therefore, histone acetylation does not always correlate with a normal phenotype. Localized hyperacetylation of genes involved in oncogenesis triggered by hormones can precipitate a neoplastic phenotype.
7.7 LUNG AND NASAL CANCERS MEDIATED BY HISTONE ACETYLATION

7.7.1 NICKEL EXPOSURE AND HISTONE ACETYLATION

Occupational exposure to soluble nickel is known to cause lung and nasal cancers [17]. Besides DNA damage and slow repair response, nickel affects chromatin configuration [17]. Nickel has been shown to decrease the acetylation of histone H3 and H4. In fact, inhibition of gene expression has been associated with hypoacetylation of histones H3 and H4 as a result of nickel-mediated inhibition of HAT activity [17]. Nickel induces an overall decrease in acetylation of all histones. In the group of histones, H2b is the most sensitive to deacetylation and H3 is the least sensitive. Lysines at position 5, 12, 15, and 20 are affected in H2b [17]. It has been observed though that the sensitivity to deacetylation of H2b is related to the exposure periods and shorter exposures have no profound effect on the acetylated states of H2b K5 and K15 residues. K12 and K20 however undergo a different fate, and directly correlate to the time and dose of nickel exposure [17]. Therefore genes containing residues that are primarily acetylated at these positions will be affected and would induce a cascade of downstream effects. In most instances hyperacetylation is associated with gene expression, however, some studies have shown that acetylation of histones can inhibit expression. This inhibition is primarily caused by a blend of hyper/hypoacetylated patterns at specific lysine residues on different histone subunits at promoter and coding locations. These patterns are more gene-specific and do not associate with the overall global hypo-or hyperacetylation states.
7.7.2 LUNG CANCERS ASSOCIATED WITH ACETYLATION OF THE *RAR\beta* GENE

In most lung cancers, the *RAR\beta* gene is defective and therefore is not expressed in the presence of retinoic acid (RA) imparting a retinoic acid refractory phenotype to the cells [18]. Analysis of lung cancer cell lines have shown that cell lines unresponsive to RA have deacetylation of histone H3 with hyperacetylation of histone H4 [18]. However, in RA-responsive cells, both H3 and H4 are acetylated at the promoter of RARb receptor, irrespective of the methylation status of the promoter [18]. Therefore, a reduction in H3 acetylation has been shown to correlate with the RA refractoriness in lung cancer cells [18]. In addition to reduced histone acetylation at specific subunits, methylation of a promoter may be a secondary essential mechanism to gene expression. Therefore, some lung cancers are associated with hypoacetylation and hypermethylation of specific moieties that control the expression of *RAR\beta*, which is crucial for downstream signaling pathways.

7.8 MODULATION OF HISTONE H4 IN GASTRIC CANCERS

In a majority of gastric and colorectal cancers H4 acetylation is markedly reduced which precipitates invasive and metastatic events [19]. In colon cancers, global hypoacetylation enhances tumor invasiveness and metastasis allowing for a means of possible cancer therapy. Gastric carcinomas are of two types, intestinal and diffuse [19]. In both these types of cancers deacetylation of histone H4 is observed. This reduction is gradual and begins from the early stage where precancerous lesions predominate and progress into the late stages of invasiveness and metastasis [19, 20]. However, one should be careful in
interpreting the observed results as acetylation alone cannot dictate a cancerous phenotype but relies on a multitude of factors and mechanisms, all of which target genes that control normal cell phenotype.

7.9 CANCERS OF THE THYROID AND HISTONE ACETYLATION

Essentially the process of acetylation or deacetylation of histones are not directly involved in neoplastic transformations. It is a domino effect induced by modulations of transcription factors, which control cell-specific functions that influence oncogenic events. For instance in many thyroid cancers the sodium iodide symporter (NIS) expression is downregulated [21]. The expression of this protein, NIS, is important as it is involved in iodine uptake. In primary solid thyroid tumors the cells are unable to absorb iodine in part due to low or no expression of NIS [22]. Reduced acetylation in conjunction with methylation at the NIS promoter is probably responsible for the decreased NIS expression [21]. Studies have shown that histone reacetylation by inhibition of the deacetylation process stimulates NIS expression [21, 22]. Thus, targeting enzymes that inhibit histone deacetylation may have potential as a therapeutic measure against thyroid cancers. This process helps not only in target-specific treatments but also in the induction of differentiation and apoptosis by increasing the expression of cell cycle regulatory genes.

RA treatment is another approach to restore radioactive iodine uptake in a small subset of metastatic thyroid cancers [22]. The promoter region of RARb, a receptor for RA, appears to be unmethylated and deacetylated at H3 and H4, but one must be cautious to interpretations, as these observations are cell-type specific. In the presence of the
HDACs alone or in combined treatments with RA, the acetylation levels can be restored. Therefore alterations in histone patterns can contribute to the refractoriness in differentiated thyroid cancers and may be a suitable tool for therapy. This study highlights that acetylation is not responsible for the direct conversion of a normal cell to neoplastic forms but controls a host of other genes crucial for downstream effects.

7.10 GENES AFFECTED BY ACETYLATION THAT PLAY A ROLE IN CARCINOGENESIS

A myriad of genes are associated with cancers. In this section, we discuss a few genes that are affected by modulations of histone molecules. These modulations may affect gene expression in several ways: (1) directly affect the promoter region of the genes, (2) affect coactivators or transcription factors that bind to promoter regions, and (3) affect the expression and stability of proteins that bind to other coactivators. We have discussed those genes whose expression is crucial to various stages of the cell cycle (Figure 7.3) and the expression of which is completely deregulated in a majority of cancers. Deregulation in any of these phases by changes in regulation of genes controlling these phases can lead to a cancer phenotype.

7.11 p53 AND p21

Cyclins and cyclin-dependent kinase genes control the transition of cells in each of the G1, S, and G2 phases of the cell cycle and are essential as they direct cell division in a regulated manner. The cell cycle is constantly monitored and a close surveillance is kept
FIGURE 7.3 Genes that are regulated by histone modulations across the cell cycle. Acetylation can affect any of the genes or pathways depicted above. Hypoacetylation of genes that induce apoptosis can induce the cell to enter the S phase for continued growth. Similarly, hyperacetylation of genes required for the S and G phases of the cell cycle can result in uncontrolled growth resulting in transformations and sustained tumor growth.
on damage to DNA. When DNA damage is detected, proteins involved in rectifying the damage are induced. These proteins force the cell to exit the cell cycle and undergo apoptosis. p53 and p21 are two very important proteins required for this function. p21 comes under the direct control of p53; however, p21 expression can be induced by p53-independent pathways as well. The induction of p53 stimulates the production of p21 that is a cyclin-dependent kinase. This protein inhibits cdk4/cyclinD and the downstream pathways associated with it resulting in cell cycle arrest.

Transactivators not only have the ability to induce gene expression but some of them, such as PCAF and p300 posses intrinsic HAT-like activity. [23]. In most cases histones associated with promoter regions are susceptible to HAT activity, which alters the residues to modulate expression. In some instances HATs associate with proteins directly and modulate downstream effects. Acetylation of lysine residues in proteins can enhance their binding ability to DNA domains. The affinity to the binding site, which could be a DNA binding domain or a protein domain, is important, because this affinity dictates the exponential downstream effects responsible in maintaining a structurally and functionally stable cell. Such is the case with the protein p53 in that its transcriptional regulation is not directly mediated by acetylation, but the protein itself is acetylated to enhance its stability and binding affinity to its consensus DNA binding regions [23].

p21 can be induced by p53 dependent and independent pathways. Studies have shown that acetylation of p53 specifically at residues K373/382 is important to induce p21 expression [12]. This induction is independent of the phosphorylation of Ser residues at 15, 20, and 392. In the majority of cancers p53 expression is reduced or completely inhibited. p53 regulates its expression via a feed back loop mechanism. Thus, in the
absence of p53 or in the presence of deacetylated p53, the tumor suppressor is unable to
bind to the p21 promoter thereby attenuating the expression of p21. Acetylation of
specific residues is necessary to bring about this effect. Acetylation of K 373/382 may
bring about a conformation change that increases the binding affinity and specificity of
the p53 molecule; however, other modulations may reverse this affinity. If p21 is no
longer expressed then the apoptotic pathways controlled by this protein are affected and
the cells escape this check and proceed toward a proliferating phenotype. In some
malignancies histones conjoining at the promoter of p21 are hypoacetylated, inactivating
expression [24]. Therefore, inhibition of p21 expression mediated by hypoacetylation of
its promoter or hypoacetylation of the p53 protein can be one of the early mechanisms
directing cells toward a more cancer-like phenotype [24–26].

7.12 hTERT

hTERT, the catalytic subunit of telomerase, is upregulated in the majority of cancers and
p53 and p21 (a p53-induced protein) are downregulated. Many studies have focused on
the promoter region of hTERT to determine factors that govern its expression since
hTERT is the rate-limiting message for telomerase activity. DNA methylation and
histone modulations, primarily acetylations, have been linked to regulation of hTERT
both in normal as well as malignant cells [27-31].

In most normal cells, with the exception of the germ cells and adult stem cells as
well as a few other cell types such as those of the intestinal lining, hTERT is absent. In
normal cells, hTERT repression is mediated by negative modulators, which when bound
to the hTERT promoter can induce deacetylation of the promoter, primarily at histones
H3 and H4 [30]. The hTERT promoter has binding sites for Mad1, a repressor protein by function. It is believed that Mad1 modulates the expression of hTERT via the deacetylation of the histones that complex to the promoter [27,30]. Mad1 binds to the mSin3 corepressor protein that has deacetylase activity. Thus, when these repressor complexes bind to their DNA binding regions at the hTERT promoter, the deacetylation switch is turned on and the histones surrounding the promoter are deacetylated [30]. With deacetylation, changes in histone and chromatin dynamics of the hTERT promoter ensue, which prevent oncogenic activators of hTERT from binding to their DNA-binding domains. This change in histone dynamics may not entirely be brought about by histone acetylation but also by other modulating processes like methylation of CpG islands and specific lysine residues of histone protein with the hTERT promoter.

In some cases, normal cells tend to escape the stringent cell cycle checks and instead of following the senescent or apoptotic pathways, proliferate by the induction of the repressed hTERT and reactivation of the telomerase molecule. The reactivation of hTERT has been linked to many mechanisms [30]. Recently though, epigenetic mechanisms are found to be the hallmark in hTERT control [30]. In certain malignancies reactivation of hETRT has been linked to the phosphorylation of Ser 10 of the histone H3 subunit via the p38 mitogen-activated kinase pathway [32]. However, this transactivation is dependent on the acetylation of the H3 K14 residue [32]. Histone modulations sometimes function using various histone marks and this is one such example. Ser 10 phosphorylation alone weakly induces hTERT expression and telomerase activity. However, only when the Ser 10 moiety of H3 is phosphorylated with the combined acetylation of H3 K14 at the hTERT promoter is the hTERT specifically transduced. This
occurrence may be cell-specific but is proof to the epigenetic mechanisms of hTERT control.

Interpretations of the effects of histone acetylation on gene expression should be made carefully as histone modulations may be global but in most tumorigenic cases local hyperacetylation of oncogenes, such as in the case of HPVE6 is observed. The HPVE6 protein is responsible for a majority of virally transduced cervical cancers. Interestingly this protein is also know to be a modulator of hTERT expression and has a DNA-binding domain on the promoter. The E6 protein modulates the expression of hTERT, the rate-limiting molecule of telomerase, by the acetylation of histone H3 of the hTERT promoter in association with E6-associated protein [33].

7.13 cMYc TRANSCRIPTION ACTIVATOR

cMyc is an oncogene and is notably overexpressed in many tumors including breast, colon, and prostate. About 5% of the total of genes studied so far are affected by cMyc [34]. The mechanism of cMyc-mediated target gene expression is not yet fully understood. Like many other transcription factors, Myc has the ability of affecting target gene expression directly by binding to its promoter residing domains. Indirect pathways can include sequential or combinatorial protein interactions [35]. Myc has the ability to acetylate histones, especially H4 and H3 [35,36] and cMyc interacts with transactivators like TRRAP, a protein that tethers to the GCN5 and Tip60 proteins that have HAT activity [35,36]. Acetylation of cMyc target genes is modulated by this large protein complex; however, actual transactivation of the promoters of its target genes is dependent on other mediators as well [34,35]. Histone acetylation mediated gene expression by
cMyc, however, has been the subject of some debate [34]. Some studies have demonstrated that the transcriptional expression of cMyc target genes may be independent of histone acetylation but could be a very essential step in further downstream gene expressions [34,37]. Therefore when cMyc is overexpressed it can essentially cause the hyperacetylation of histones of its target genes which otherwise in normal cells are maintained at optimal acetylation/deacetylation balance. This modulation of histone dynamics can dramatically affect the expression of cMyc-mediated mitogen activated target genes.

7.14 MAD1 REPRESSOR

Mad1 is an antagonist of Myc and is involved in repressing gene expression. Both these transcription factors compete for the same binding site at the E-box as a complex with Max. This binding is seen in the transcriptional control of promoters of genes like cyclin D2, Cad, and hTERT [38]. For these genes, in quiescent cells or differentiated cells, Mad1 attenuates and represses expression by deacetylation of their promoters. This inactivation is brought about by HDACs (histone deacetylases) recruited to the promoter regions by Mad1 in tandem with corepressor Sin3 [30,38]. Deacetylation recoils the chromatin at these important promoter regions terminating expression of cancer promoting genes in conjunction with reactivation of cell cycle control genes.

7.15 Mnt COTRANSACTIVATOR OR REPRESSOR

Mnt, a transcription repressor, interacts with coactivators such as cMyc and Max and forms a regulatory network that controls downstream genes, important in fine tuning the
proliferation, differentiation, and quiescent phases of a cell. Myc and Mnt are coexpressed in proliferating cells and Mnt competes with Myc to dimerize with Max and bind to the E-box elements [39,40]. However Mnt initiates the induction of Myc target genes such as cyclin D2 important for the S phase of the cell cycle [39]. It has been documented that the functions of Mnt are mediated by the association with corepressors Sin3, which recruits HDAC to deacetylate histones [39]. Mnt functions in repressing Myc-mediated tumor formation as well as the activation of Myc-targeted promotors that contain E-boxes. Therefore Mnt repressive or activating function is perhaps regulated by many mechanisms but fluctuations in histone dynamics is a plausible explanation. Thus, when the functions of this transcription factor is de-regulated, either by mutations or other such changes, its downstream histone modulations are affected inducing the overexpression of Myc or other such targets resulting in the development of neoplasms.

7.16 Sp1 COACTIVATOR

Sp1 modulates gene activation or repression. This action is solely dependent on the target transcription factor to which it binds. For example Sp1 influences the regulation of MCP1 (monocyte chemoattractant protein 1). The MCP1 protein is a chemokine that is involved in recruiting monocytes/macrophages to the areas of inflammation. However, this protein is also linked to prostate neoplasia and prostate adenocarcinomas. Tumor necrosis factor (TNF) modulates the expression of MCP1 and does so by recruiting acetylases CBP/p300 to the promoter regions. This chromatin-modulated induction of MCP1 requires the activity of p65 and Sp1. TNF action acetylates both the proximal and distal regions of MCP1 promoter in conjunction with p65 activity, but Sp1 acetylates only the distal
regions of MCP1. p65 and Sp1 perhaps only aid in the regulation of gene activity but may not be involved with direct induction of the gene. Such an observation is interesting as binding sites for Sp1 are present on other transcription factor promoters and genes associated with cancers such as hTERT. Any disturbance in the genes involved in regulating gene activity by acetylation may generate imbalances in histone acetylation patterns. This may lead to the induction of continuous oncogenic expression in conjunction with the reduction of cell cycle regulatory proteins.

7.17 FUTURE PROSPECTS

Many genes are important in orchestrating the fine balance between cell proliferation versus tumorigenesis. Even if it is not the primary mechanism of gene control, this balance is mediated by chromatin fold changes via histone modulation. For a given gene expression, several pathways may function in tandem to bring about the expression. Failure in any step along the pathway can have detrimental effects, which is seen in many cancers. This failure occurs when the nucleosome units undergo aberrations, resulting in changes in the histone code and affecting the fluidity of DNA regions blocking access to binding elements. Therapeutics may have potential in utilizing the compounds that can reverse the abnormal histone code bringing the functions to normal.

REFERENCES

DIETARY AND ENVIRONMENTAL INFLUENCES ON HISTONE MODIFICATIONS IN CANCER

by

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_Cancer Epigenetics_

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11.1 INTRODUCTION

Many factors intricately regulate the expression of genes responsible for development and growth. Aberrations in their expression can result in growth and developmental abnormalities, including cancer. Initially, the understanding was that mutations in the genome, i.e., variations in the DNA sequence, were the primary reason [1–3]. However, recent advances have proven, otherwise. Epigenetic mechanisms modifying gene expression without affecting the DNA sequence are heritable and can perpetuate altered phenotypes as well.

11.1.1 CHROMATIN STRUCTURE: THE BASIS OF EPIGENETIC MECHANISMS

Chromatin is the basic organizational form of DNA in the eukaryotic nucleus. The repeat unit of chromatin is the core nucleosome in which 145 base pairs of DNA are wrapped around a histone octamer consisting of two molecules each of the core histones H2A, H2B, H3, and H4. Core histones are susceptible to enzymatic modifications of their tails. The tails that protrude out of the complex are primarily lysine, arginine, and serine residues which can be acetylated, methylated, ubiquitinated, biotinylated, phosphorylated, and sumoylated to influence many different functions pertaining to gene expression and transcriptome stability [4,5].

Nucleosomal arrays along the DNA are believed to fold into a 30 nm fiber upon incorporation of the linker histone H1 [5]. Given its folded structure, chromatin does not generally allow extraneous access. Ionic interactions between the charged DNA and histones have to be overcome to unfold the chromatin structure and allow access of the transcription machinery to the DNA and, finally, gene expression. Moreover, the original
chromatin structure has to be reinstated after the tasks have been completed. These chromatin-expression states can be maintained through multiple rounds of cell division that occur during development [6–8]. Given their nature, these chromatin states are prone to intrinsic, intracellular, as well as external cues such as diet, environmental carcinogens and pollutants, resulting in cells with identical DNA sequences but phenotypic differences between them.

11.1.2 TYPES OF EPIGENETIC MODIFICATIONS

The most widely investigated and understood epigenetic changes that contribute to altered gene expression are genomic methylation of CpG islands, histone modifications, and RNA-associated silencing [5–9]. These modifications seem to be interpreted by proteins that recognize specific modifications and facilitate the appropriate downstream effects. Such modifications which influence normal development, growth, and aging can, under certain conditions, initiate diseased states.

Epigenetic imprinting, established during gametogenesis, is faithfully passed on from one generation to the next [10]. A wide variety of dietary components including grains, legumes, fruits, vegetables, tea, and wine which consist mostly of isoflavones possess anticarcinogenic activities [11]. Although the antioxidant and antiproliferative activities of these compounds have been attributed to the action of specific phytochemicals, evidence suggests that the optimal anticarcinogenic effect is obtained by consuming the complex food containing them rather than the isolated putative active ingredients. Section 11.3 discusses epigenetic mechanisms and the influence of diet on each of them.
11.2 GENOMIC METHYLATION OF CPG ISLANDS AND THE DIET

The activity of DNA methyltransferase 1 (DNMT1), an enzyme which methylates CpG residues, is affected by the level of S-adenosylmethionine (SAM). SAM function requires the availability of the methyl moiety, the absence or presence of which can affect DNMT1 activity. The source of the methyl moiety is in methyl-rich diets. Thus, the methyl-content of the diet may affect the activity of DNMT1, methylation of CpG islands and, indirectly, the epigenomic code.

The reversible histone-tail modifications discussed in Section 2.1 can result in higher or lower affinity interactions between DNA and core histones, inducing silenced or activated states of gene expression, respectively. Moreover, they can also affect the modification of other histones to co-mediate effects on gene expression (Table 11.1) [5]. Bioactive molecules and nutrients derived from dietary sources, such as dairy products, soyabean, vegetables, and green tea [10–13] are known to mediate such effects (Figure 11.1).

11.3 HISTONE MODIFICATIONS

11.3.1 HISTONE ACETYLATION/DEACETYLATION AND THE EFFECT OF DIET

Acetylation of the ε amino group of the lysine residues of histones neutralizes their positive charge and reduces the strength of the interaction between DNA and the acetylated histones thereby leading to chromatin unfolding and euchromatin formation.
Acetylation, phosphorylation, and glycosylation of histones are mostly associated with euchromatin formation whereas methylation, sumoylation, ADP-ribosylation, and biotinylation of histones are mostly associated with heterochromatin formation. Diallyl sulfide and butyrate act as histone deacetylase (HDAC) inhibitors in part by decreasing c-myc levels and increasing p21 levels. Flavones and sulforaphanes are also HDAC inhibitors, while curcumin inhibits histone methyltransferases (HMT). Polyphenols such as EGCG and genistein inhibit HDACs by downregulating DNMT1 associated with increased levels of S-adenosyl methionine. Methyl deficient-diets result in hypomethylation by inhibiting histone methyltransferases (HMT). Nickel at very low concentrations causes mono-ubiquitination of histones which in turn inhibits HMT. Vinblastine, zearalenone, and genistein (at >30 mM/L) increase kinase activity and promotes histone phosphorylation while genistein (at 2–20 mM/L) causes hypophosphorylation of phosphorylated histones (p histones) associated with angiogenesis.
<table>
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<tr>
<td>H3</td>
<td>Lysine</td>
<td>79</td>
<td>Methylating</td>
<td>Euochromatin</td>
<td>[5,158]</td>
</tr>
<tr>
<td>H3</td>
<td>Lysine</td>
<td>9</td>
<td>Mono-, di-, and trimethylation</td>
<td>Trimethylation mediates transcriptional silencing</td>
<td>[5,99,158]</td>
</tr>
<tr>
<td>H3</td>
<td>Lysine</td>
<td>9</td>
<td>Methylating</td>
<td>Heterochromatin; Inhibits methylation of H3 K4</td>
<td>[5,99]</td>
</tr>
<tr>
<td>H3</td>
<td>Lysines</td>
<td>27, 36</td>
<td>Mono-, di-, and trimethylation</td>
<td>K27 methylation involved in X chromosome inactivation; K36 methylation is also target for acetylation and functions as a chromatin switch in the regulation of gene expression</td>
<td>[5,159,160]</td>
</tr>
<tr>
<td>H4</td>
<td>Lysine</td>
<td>20</td>
<td>Mono-, di-, and trimethylation</td>
<td>Associated with heterochromatin domains</td>
<td>[5]</td>
</tr>
<tr>
<td>H3</td>
<td>Arginine</td>
<td>8, 17</td>
<td>Methylating</td>
<td>Possibly involved in gene activation. Further studies required to clarify the role. H3 R8 methylation may be blocked by H3K9 acetylation</td>
<td>[5,126,161,162]</td>
</tr>
<tr>
<td>H4</td>
<td>Arginine</td>
<td>3</td>
<td>Methylating</td>
<td>Role not clear</td>
<td>[5]</td>
</tr>
<tr>
<td>H3</td>
<td>Serine</td>
<td>10</td>
<td>Phosphorylation</td>
<td>Inhibits methylation of H3-K9</td>
<td>[5]</td>
</tr>
<tr>
<td>H3</td>
<td>Lysines</td>
<td>9, 14</td>
<td>Acetylation</td>
<td>Associated with euchromatin; Activates phosphorylation of H3-S10</td>
<td>[5,158]</td>
</tr>
<tr>
<td>H3</td>
<td>Lysines</td>
<td>5, 10, 20</td>
<td>Biotinylation</td>
<td>May be involved in DNA repair, cell cycle proliferation</td>
<td>[99]</td>
</tr>
<tr>
<td>H2A</td>
<td>Lysines</td>
<td>10, 15, 125, 127, 129</td>
<td>Biotinylation</td>
<td>Role not clear</td>
<td>[99]</td>
</tr>
<tr>
<td>H4</td>
<td>Lysines</td>
<td>8, 12</td>
<td>Biotinylation</td>
<td>Involved in heterochromatin structures and gene silencing</td>
<td>[99]</td>
</tr>
</tbody>
</table>
As a result, histone acetylation usually causes transcriptional activation [5]. Histone acetylation is regulated by the action of two antagonistic enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Interestingly, inhibitors of these enzymatic activities interfere with the cell cycle, at least in part by triggering changes in the acetylation/deacetylation balance of histones. This induces growth arrest, differentiation, and apoptosis [14].

Several dietary ingredients known to possess anticarcinogenic activities [12, 15] have the ability to inhibit HDAC and HAT enzymes. For example, butyrate (Figure 11.1), generated in the colon by the fermentation of dietary fiber, is a histone deacetylase inhibitor. Butyrate promotes acetylation of histones, leading to expression of genes involved in cellular differentiation and apoptosis of cancer cells [16]. Further supporting the link between histone acetylation and the activation of gene expression, butyrate increases the expression of the CDKNIA gene in human colon adenocarcinoma (Colo-320) and human colon cancer (SW116) cell lines, inducing expression of the p21 protein. Interestingly, the latter is due to the hyperacetylation of H3 and H4 histones associated with the promoter of the p21 gene [14].

Flavones are potent cell-specific triggers of apoptosis in a variety of cells [17–45]. Flavopiridol, a flavonoid derived from an indigenous plant from India, induces apoptosis in human leukemia cells by the disruption of cell cycle progression. The result is more pronounced when flavopiridol is used in combination with sodium butyrate as demonstrated in U937 leukemia cells [46]. In combination, these compounds inhibit leukemic cell differentiation and promote mitochondrial damage and cell death by the induction of multiple perturbations in cell cycle and apoptosis regulatory proteins, further
supporting the possibility that butyrate effects on histone acetylation may be necessary for an optimal anticarcinogenic effect [47].

The Coxsackie and adenovirus receptor (CAR) is downregulated in several types of cancer [48–55]. Phytoestrogens in combination with FK228, a depsipeptide HDAC, increase the expression of CAR. Pong et al. [54] have reported the potency of phytoestrogen in combination with FK228 to be: genistein>biochanin A>ipriflavone>daidzein. Interestingly, this order correlates with the degree of acetylated H4 levels associated with the CAR promoter, supporting the possibility that the combined effect of phytoestrogens and FK228 on the expression of CAR is regulated by histone acetylation [47].

Diallyl disulfide (DADS), present in garlic and other allium vegetables, has been shown to induce G2/M cell cycle arrest in HT-29 and Caco-2 human colon cancer cell lines and this effect also seems to be mediated by the state of histone acetylation. Thus, treating these cells with 200 mM DADS increases CDKNIA mRNA expression and p21 protein levels, accompanied by an increase in H4 or H3 acetylation within the CDKNIA gene promoter region. Increase in H4 or H3 acetylation patterns are also observed in vitro with other organosulfur compounds found in garlic such as S-allyl-mercaptopcysteine [56–60].

Sulforaphane, a compound found in cruciferous vegetables, acts in vitro as a HDAC inhibitor in the range of 3–15 mM, producing an increase in acetylation of histones in human embryonic kidney 293 cells, HCT116 human colorectal cancer cells, and prostate epithelial cells lines (e.g., BPH-1, LnCaP, and PC-3). Cells treated with histone deacetylase inhibitors have been shown to have increased acetylation of histones
that specifically regulate genes involved in differentiation and apoptotic pathways [61–65]. HDAC inhibitors work by inactivating the deacetylases thereby changing the acetylation/deacetylation balance and increasing the expression of genes that they control.

Although the molecular pathways that are affected by HDACs are poorly understood, in most cancer phenotypes the use of HDAC inhibitors are associated with the induction of cell differentiation, apoptosis, and death of the tumor cells [66]. For example, induction of G2/M cell cycle arrest and apoptosis by HDAC inhibitors, e.g., sulforaphane, correlates with elevated expression of p21, a protein known to be involved in cell cycle arrest and apoptosis, [16,61,63–64]. Probably the acetylation levels of histones, in this case histone H4 associated with the p21 promoter, increases [63–64]. Furthermore, increased acetylated levels of histones H3 or H4 were found in intestinal polyps of Apcmin mice treated with sulforaphane, which also displayed increased p21 expression and decreased multiplicity of the polyps when compared to the Apcmin mice fed with a control diet without sulforaphane [58,63].

Curcumin (diferuloylmethane) (Figure 11.1), an active ingredient derived from the rhizome of the plant Curcuma longa, has also been shown to have anticancer activity both in vitro and in vivo. At 20 mM, curcumin induces histone hypoacetylation by inhibiting HAT activity in brain cancer cells in vitro [67]. The inhibitory effect of curcumin on cell proliferation was associated in a time- and concentration-dependent manner with histone acetylation [67]. Interestingly, curcumin upregulates or downregulates histone acetylation, depending on the cell type. For example, in brain cancer cells treated with curcumin, H4 histones are hypoacetylated [67]. In contrast, H3
and H4 histone subunits are hyperacetylated in androgen-dependent and -independent prostrate cancer cells treated with curcumin [68]. This cell-specific effect may be attributed to different genes affected downstream of the histone modification in each of these cell types. Alternatively, the opposite findings may be due to the different concentrations of curcumin used in the two studies.

Recently, a small polyisoprenylated benzophenone molecule, garcinol from the Garcinia indica (coccum) fruit rind, has been found to be a naturally occurring inhibitor of HAT [68]. Therefore, it is possible that treatment of cells with garcinol may induce hypoacetylation by inactivating HAT. Further studies will be necessary to determine the efficacy of using compounds that target HAT activity.

11.3.2 HISTONE METHYLATION/DEMETHYLATION AND THE EFFECT OF DIET

11.3.2.1 Histone Methylation

Evidence exists that histone methylation regulates fundamental processes such as heterochromatin formation, X chromosome inactivation, genomic imprinting, transcriptional regulation, and DNA repair [69]. The most heavily methylated histone is H3, followed by H4. Histone methylation is a covalent modification that occurs on the side chain nitrogen atoms of key histone lysines and arginines. Arginine can be either mono- or dimethylated and lysine can be mono-, di-, or trimethylated. The multiple possible states of methylation of a particular histone residue, determined by different histone methyltransferases [5,70–71], leads to different biological outcomes; thus, histone methylation has greater combinatorial potential as compared to other histone modifications.
Trimethylation of the histone H4 lysine 20 residue (H4-Lys20), the only lysine that undergoes methylation in H4, has been associated with constitutive heterochromatin, gene silencing, and aging, and serves as a marker for them [69,72]. Fraga et al. observed that cancer cells have a loss of trimethylated forms of histone H4 [72]. This loss occurs early and accumulates during the carcinogenic process in the skin cancer model with which Fraga worked. The resulting overall hypomethylation may be responsible for gene silencing observed at various stages of carcinogenesis [72].

Further evidence for the involvement of histone methylation in cancer was provided by studies in mice, in which knockout of the enzymes that govern H3-Lys9 methylation resulted in genomic instability and formation of B-cell lymphomas in 28% of the mice between 9 and 15 months of age [73].

11.3.2.2 Histone Demethylation

The removal of a methyl group from histones is achieved by arginine and lysine demethylases, e.g., by deimination and amino-oxidase reactions [69,74]. In contrast to acetylation or phosphorylation, which has fast turnover rates and fit the expected features of a regulatory modification, histone methylation has been demonstrated to have a slow turnover rate. Modifications such as methylation are more stable and are responsible for long-term expression status of certain regions of the genome. Active histone demethylation is involved in both either transcriptional expression or repression and depend on the histone residues that are demethylated.
11.3.2.3 Dietary Influence on Histone Methylation and Demethylation

11.3.2.3.1 Effect of a Methyl-Deficient Diet

Histone methyltransferases require methyl-rich sources to catalyze the transfer of methyl groups to histone lysines or arginines. In the normal cell, SAM, a product of methionine metabolism, serves as a methyl-donor, resulting in methylated CpG islands. Diet is a major source of methyl groups [75–77]. A diet low in methyl-donors may lead to changes in trimethylation and acetylation patterns of H4-Lys 20 and H3-Lys 9, respectively, resulting in the formation of relaxed DNA–histone complexes. Such changes have been observed during hepatocarcinogenesis [77].

Folate is an important dietary methyl-donor, which has dual effects in cancer inhibition and progression [78–79]. Folate is required for 1-C carbon metabolism as well as nucleotide synthesis, all of which are essential for cell growth. Folate is also metabolized to 5 methyltetrahydrofolate, which is a source of methyl groups for methionine and SAM synthesis.

Carcinogenesis in several organs such as colon, prostate, and lung [78,80], have been associated with folate-deficient diets. In contrast, the incidence of breast cancer has been associated with diets high in folate. The key factors that tip the balance towards tumor progression or regression are the timing and dosage of folate administered. This may be a reason that a folate-deficient diet or a diet high in folate may increase the risk of tumor incidence. DNA methylation has been associated with carcinogenesis in these organs. Determining the genes silenced by this mechanism could provide much needed markers for the diagnosis of these cancers [79]. Understanding the molecular
mechanism of folate action in different tissues is, therefore, crucial to the understanding of its chemopreventive potential.

11.3.2.3.2 Dietary Polyphenols

The catechol polyphenols inhibit DNA methyltransferases 1 (DNMT1) activity in part by increasing intracellular SAH (S-adenosyl-L-homocysteine), a potent inhibitor of DNA methyltransferases [81] (Figure 11.1). The effective in vitro apoptotic and cell proliferation inhibitory concentrations of epigallocatechin gallate (EGCG) (10–50 mM), a key component of green tea, are ~50 times higher than the plasma and tissue levels of EGCG generally observed after ingestion of tea [82–83]. The effective high in vitro concentrations may be attributed to the inactivation of bioactive metabolites of EGCG by the culture media used in studies in vitro. Therefore more of the compound is required to bring about an effective response. However, in vivo, a host of factors could be involved in stabilizing the EGCG molecule, allowing low plasma doses to be more effective. Discrepancies have also been found between the in vitro and in vivo effective concentrations for genistein and may have similar causes. For example, in vitro, genistein concentrations (5–20 mM) that trigger DNA demethylation are orders of magnitude higher than plasma levels of genistein (~270 nM) [84–85].

11.3.3 HISTONE PHOSPHORYLATION AND THE EFFECT OF DIET

The core histones and histone H1 undergo phosphorylation on specific serine (Ser) and threonine (Thr) residues by H1 and H3 kinases [86–87]. H1 is phosphorylated on the N-terminal and C-terminal domains of Ser/Thr residues, whereas H3 is phosphorylated only
on the N-terminal domains of Ser/Thr residues. Both phosphorylation processes are cell cycle dependent [88]. H1 phosphorylation weakens H1 binding to DNA, promoting free access of transcriptional replication factors to the DNA, which facilitates gene expression. H3 phosphorylation at Ser-10, a more frequent process, has been associated with the transcriptional activation of the early response genes c-fos and c-jun. Thus, chromatin decondensation is possibly coordinated by H3 phosphorylation [89]. These findings are consistent with the possibility that H3 phosphorylation may play a role in cell cycle progression [89].

The activation of checkpoints in response to DNA damage leads to cell cycle arrest but when the damage is very severe, it results in apoptotic cell death [90–91]. Sulforaphane, found in mustard seeds, although not an antioxidant itself, is an effective inducer of enzymes that enhance the activity of the crucial intracellular antioxidant, glutathione. In vitro, 20 mM sulforaphane increases phosphorylation at Ser-139 of H2A.X, an isoprotein of histone 2A (H2A) and is a sensitive marker for the presence of DNA double-strand breaks. The ensuing accumulation of cell cycle damage may be the mechanism that triggers apoptosis [89].

Histone phosphorylation has been associated with the anticarcinogenic activity of several flavones [92]. For example, H2A.X phosphorylation known to be induced at double-strand break sites have been observed in MCF-7 cells treated with 50 nM aminoflavones. Moreover, the aminoflavone had a dose-dependent effect on histone phosphorylation [92–94]. The ability of aminoflavone to generate phosphorylated H2A.X selectively in replicating cells makes it a suitable candidate for the treatment of cancers.
The chemopreventive effect of genistein has been associated with increased H1 histone phosphorylation in cancer cells [95]. Genistein-treated breast cancer cell lines such as MDA-MB-231 and BT20 display an increase in kinase activity. This study primarily focused on the expression of cyclin and cyclin-dependent kinases. The increase in kinase activity in genistein-treated cancer cell lines is accompanied by histone phosphorylation, transcriptional activation, and finally G2M arrest [95]. In contrast to this observation genistein also blocks the recruitment of histone H3 kinase MSK1, thereby inhibiting H3 phosphorylation. This observation is gene specific, however. Interleukin-6, an inflammatory response gene, and a growth factor for many tumors [96], is transcriptionally regulated by nuclear factor-kB (NF-kB) and requires the activation of the mitogen-activated protein kinase (MAPK)/MSK kinase pathway, which phosphorylates NF-kB p65 and histone H3. In the presence of genistein, as seen in the breast cancer cell line MDA-MB-231, histone H3 kinase MSK1 recruitment to the IL-6 promoter is blocked leading to the loss of H3 phosphorylation and acetylation and reduced IL-6 expression. However, the activity of MSK1 is reduced but not completely inhibited. Although genistein plays a significant role in H3 phosphorylation, the contradictory results obtained by the two studies was primarily due to the different pathways analyzed and the concentrations of genistein used were different (1–30 mM and 200 mM, respectively).

Zearalenone, a fungal metabolite found in dairy sources, meat, and grains, is a nonsteroidal estrogen molecule [97]. Ingestion of zearalenone increases the endogenous estrogen load thereby stimulating estrogen receptor-positive cells to undergo mitosis by
activating cyclin-dependent kinases, [97]. It is, therefore, not surprising that phosphorylation of histone H3, a marker of mitotic activity, is also increased [97].

Another molecule that influences the phosphorylation status of histones H3 is vinblastine, an alkaloid derived from the Madagascar Periwinkle plant. This compound has been used in the treatment of cancers [98]. In vitro treatment of cancer cell lines with this alkaloid shows that the proportion of phosphorylated versus nonphosphorylated H3 molecules progressively increases during the cell cycle arrest in the metaphase; but in the interphase chromatin, the fraction of phosphorylated H3 histones is several-fold lower compared to that in mitotic cells [98].

11.3.4 HISTONE BIOTINYULATION AND DIET

Recent studies have shown that histone modulations involving the covalent attachment of biotin to specific lysine residues, catalyzed by the enzymes biotinidase and holocarboxylase synthetase, occur on histones H2A, H3, and H4 [99]. Biotinylation of histone H4 at lysines 8 and 12 has been associated with heterochromatin structures, gene silencing, mitotic condensation of chromatin, and DNA repair. Histone biotinylation is a reversible process, although debiotinylases have not been characterized [99]. Biotinylation appears to be important in DNA repair and chromatin structure and is more predominant in proliferating than in quiescent cells [99,100]. Biotinylation of histones is known to occur in the presence of DNA double-strand breaks. In some cases, this occurs at specific lysine residues of H4, probably associated with genes involved in the DNA repair machinery [99]. Dietary supplementation of biotin is required for biotinylation and biotin deficiency may have profound effects on chromatin structures.
Further studies will be necessary to test the possibility that dietary biotin may have epigenetic effects via histone biotinylation.

11.3.5 HISTONE UBIQUITINATION

Recent evidence has implicated histone ubiquitination in gene transcription control [101]. Ubiquitination occurs at lysine residues of histones H2A and H2B at positions 119 and 120, respectively; H1 and H3 are not yet mapped. Mono-ubiquitination of histone H2B is required for methylation of histone H3 at K4 and K79. Histone ubiquitination seems to promote methylation by recruiting proteosomal ATPases by ubiquitin-modified H2B [102]. Nickel is an essential nutrient. However, a nickel overdose can be deleterious, as insoluble nickel compounds, and soluble nickel compounds to a lesser extent, are carcinogenic [103]. The exact mechanism by which nickel induces carcinogenesis is not clear. An interesting find is that, once in the cell, nickel compounds can exert epigenetic effects and deregulate gene expression. This is a possible mechanism underlying the etiology of nickel-induced cancers.

Nickel compounds affect three types of histone modifications (1) deacetylation of histones H2A, H2B, H3, and H4; (2) dimethylation of histone H3 at lysine 9; and (3) ubiquitination of histone H2A and H2B. At lower concentration, Ni(II) stimulates mono-ubiquitination of histones H2B and H2A while at higher concentrations ubiquitination is suppressed, apparently because of the presence of a truncated H2N which lacks the K120 ubiquitination site. This modification affects gene expression and DNA repair leading to cell transformation [104].
11.3.6 OTHER DIET-MEDIATED HISTONE MODIFICATIONS

Histone ADP-ribosylation occurs mostly on the glutamate residues of histone H2A during DNA double-strand breaks. In the thyroid, ADP-ribosylation is mainly associated with transcriptional inactivation of chromatin. Reduction of histone ADP-ribosylation is associated with various forms of cancer such as lymphoma [105]. Phosphorylation of S14 of histone H2B and poly (ADPribosylation) of glutamate residues on histone H2A are known markers of double-strand DNA breaks, which are mainly associated with transcriptional inactivation [106].

The ε-amino group of histone lysine residues is also subject to modification by ubiquitin-like proteins such as small ubiquitin-related modifier (SUMO). In contrast to histone ubiquitination, histone modifications by SUMO are generally associated with decreased gene expression, and are reversible. Sumoylation occurs on histones H2A, H2B, H3, and H4 and blocks histone acetylation and ubiquitination [107].

Histones have been reported to be glycosylated. O-linked N-acetylglucosamine (O-GlcNAc) is thought to act in a manner analogous to protein phosphorylation. O-GlcNAc additions on nuclear and cytosolic proteins possess intrinsic histone acetyltransferase (HAT) activity in vitro [108–110]. Further studies are necessary to elucidate the mechanisms and the relevance of diet-related glycosylation of histones in cancers.

In vivo histone carbonylation occurs to variable extents on all histones except histone H4. In contrast, in vitro, carbonylation has been observed on all histones, including histone H4. Histones H1 and H2A/H2B are predominantly more carbonylated than histone H3. Carbonylation is irreversible and addition of this moiety to histones rich
in basic amino acids, like lysine and arginine residues, could mask the positive charge of the nucleosome-DNA complex. This may allow the chromatin to relax, a phenomenon that normally allows gene expression. Interestingly, carbonylation of histones decreases with age, but caloric restriction has been shown to increase the levels of carbonylation [111].

11.4 EFFECT OF ENVIRONMENTAL FACTORS ON EPGENETIC MODIFICATIONS

As shown above, diet, which may be considered an “environmental factor,” is a major trigger of epigenetic modifications. Plants and animals are the major source of nourishment to humans. Therefore, the conditions in which plants and animals are reared affect the human diet. For example, if the soil in which crops are grown has a high metal content or is sprayed with pesticides that are not easily degradable, the latter make their way into the foodchain, resulting in detrimental changes to humans, including effects on the epigenome. Below, we analyze a few environment-mediated mechanisms that are well studied and are proven to contribute to epigenetic changes in cancer.

11.4.1 POLLUTANTS: SMOKING AND PARTICULATE MATTER

11.4.1.1 Mechanisms Underlying the Effect of Cigarette Smoke and Other Particulate Matter

Lung and throat cancers, as well as other respiratory problems such as chronic obstructive pulmonary disease (COPD), ensue due to chronic exposure to cigarette smoke [112–113]. The molecular pathways underlying inflammation of these tissues by cigarette smoke is
well studied. [113–114]. One of the most influential factors mediating this response is histone modification of proinflammatory genes [113–114].

Particulate matter in the air of <10 mM (PM10) diameter have also been associated with chronic lung and cardiovascular disease, including severe asthma attacks [115–121]. Just like cigarette smoke, the mechanism underlying their deleterious effect involves acetylation of H4 associated with the promoter region of inflammatory genes, e.g., IL-8 [112].

Cigarette smoke induces oxidative stress, recruits proinflammatory cells [113], and causes the overexpression of proinflammatory genes such as NF-κB and AP-1 by affecting the balance of histone-tail acetylation/deacetylation [113]. Inflammatory molecules such as interleukins and cytokines produced by the inflammatory cells induce and maintain the chronic inflamed condition [112–113]. Prolonged inflammation of the lungs increases the risk of developing COPD, which can give rise to lung cancers [122]. The mechanistic action of cigarette smoke in the induction of COPD has been investigated and chromatin changes have been implicated in its etiology. Acrolein, a reactive aldehyde, is a component of cigarette smoke which has also been shown to have epigenetic effects by interfering with the function of enzymes such as histone deacetylases (HDACs) [114].

Of the HDAC class of histone deacetylases, HDAC2 appears to be an important deacetylase involved in the pathogenesis of COPD. Studies have shown that levels of HDAC1 remain unaffected but HDAC2 levels are restored to normal after prolonged exposure to smoke [112]. Although HDAC2 levels are restored, hyperphospho-acetylation of H4 and acetylation of H3 is observed. This may be possible as the HDAC2
leve, though at optimal levels, may be inactive due to the effect of the reactive 
aldehydes present in the smoke component. These aldehydes have affinities for histidine 
and lysines, and react with the histidine groups at the HDAC2 active site. Therefore, 
drugs that are anti-inflammatory may reverse the acetylation status of the genes by 
increasing HDAC levels and activity [113–114].

Another example of histone acetylation/deacetylation imbalance induced by 
cigarette smoke, leading to altered gene expression, has been demonstrated for the 
inflammatory genes TNF-α and IL-8 mediates [112–113]. Cigarette smoke leads to 
hyperacetylation of the histone H4 associated with the promoters of TNF-α and IL-8 
[121,123]. Thus, under normal conditions, NF-κB is complexed with HDACs. A 
component of NF-κB, p65, has HAT-like activity. When complexed with HDAC, the p65 
component of NF-κB is rendered inactive and hypoacetylation of histones ensues 
[113,121]. In contrast, under conditions of oxidative stress, NF-κB is released from the 
HDAC complex. NF-κB becomes phosphorylated at serine residues, which transactivates 
p65 and leads to the hyperacetylation of histone H4. These events trigger the expression 
of the proinflammatory genes IL-8 and IL-6 [113,114,121].

11.4.1.2 Factors Modulating the Magnitude of Epigenetic Effects of Cigarette Smoke and Other 
Particulate Matter
It is presumed that individuals who smoke are at a greater risk of developing various 
pulmonary diseases as discussed above. However, not all smokers develop lung cancers 
or other respiratory problems. The reason for the susceptibility of some but not all 
smokers is not well understood. This question was addressed in a Korean population of
smokers versus nonsmokers [124]. This study provided strong evidence for a role of epigenetics in lung cancer development in susceptible populations.

Single nucleotide polymorphisms (SNPs) could be an alternative mechanism that modulates the epigenetic effects of cigarette smoke. The histone methyltransferase, SMYD3, methylates histone H3 at the K4 residue [124]. Single nucleotide polymorphisms of the gene SMYD3 are associated with greater risk for developing colorectal cancers, hepatocarcinomas, and breast cancers [124]. It is possible that polymorphisms may cause structure and activity modification of the catalytic domain of this enzyme, affecting the methylation status of histone residues. This would be likely to affect heterochromatin states and gene repression.

Yoon et al. [124] showed that SUV39H2, a mammalian histone methyltransferase, exhibits eight different SNPs. The polymorphism G to C at 1624 in the 3’ UTR is critical to the development of lung cancers. This enzyme methylates histone H3 at K9 and is involved in transcriptional repression. The addition of the methyl moiety to the K9 residue creates a binding site for heterochromatin protein 1 (HP1) [124]. SUV39H2 and HP1 both mediate the repressive functions of Rb protein that is important in controlling the cell cycle through cyclin E [124]. Therefore, histone modification and the enzymes that modulate the covalent modification may act in tandem to influence gene expression and the susceptibility to cancer.
11.4.2 OCCUPATIONAL EXPOSURE TO POLLUTANTS, HISTONE MODIFICATION, AND CANCER

In mines and refineries, workers become exposed to harmful particulate or soluble metal ions [125]. This occupational hazard has been associated with increased susceptibility to cancer. For example, occupational exposure to insoluble nickel compounds plays a significant role in the development of lung and nasal cancers [125]. When insoluble nickel enters cells it is converted to Ni$^{2+}$ ion, which induces the production of reactive oxygen species (ROS), and may repress tumor suppressor gene expression [125]. One pathway underlying Ni$^{2+}$ effects on gene expression is epigenetic. Ni$^{2+}$ inhibits the activity of histone acetyltransferases (HATs), causing histone hypoacetylation primarily of the H4 subunit. Ni$^{2+}$ binds to the histidine residue at position 18 of the amino terminal domain of H4, which is in close proximity to the acetylated lysine residue [125]. This triggers the generation of ROS which associate with HATs and may interfere with their catalytic activity, inducing hypoacetylation of associated genes. In addition to inactivating HATs, Ni$^{2+}$ bound to the histidine residue may prevent HAT binding to the H4 subunit and may consequently induce H4 hypoacetylation, which silences gene expression due to the induction of heterochromatin states [125]. ROS have a strong affinity for proteins rich in cysteine and histidine residues [125]. The catalytic domains of certain proteins, which posses HAT activity, are rich in these residues. When these HATs undergo oxidative modifications, mediated by ROS, the proteins are rendered inactive and thereby reduce histone acetylation leading to transcriptional suppression of genes associated with tumor suppression.

11.4.3 EXPOSURE TO DRUGS AND CHEMICALS, HISTONE MODIFICATION, AND
CANCER

In the late 1940s to the early 1970s expectant mothers, especially in their first trimester, were given diethylstilbestrol (DES). This synthetic estrogen was given to prevent miscarriages. Moreover, animal livestock and cattle were fed with DES to increase growth rate, providing the human population with another source of this estrogenic compound. Since then, DES has been shown to induce developmental defects of the cervix, uterus, and vagina, as well as rare cancers. The observed effect is trans-generational in nature, i.e., it skips the mothers and affects the daughters and granddaughters. This outcome is speculated to have a basis in epigenetic processes [126].

Some DES-mediated effects are mediated by DNA methylation and in certain instances methylation of CpG residues affects the modification of histones surrounding the genes. DES is a synthetic estrogen molecule. In the absence of the DES ligand or estrogen, the estrogen receptor (ER) is complexed to heat-shock protein 90 (Hsp90), and is inactive because Hsp90 inhibits ER-chromatin complex formation [126]. However, in the presence of DES, ER dimerizes with DES and enters the cell nucleus to mediate its downstream effects [126].

Steroid hormones effect the expression of WNT genes, which are involved in the development and regulation of the female reproductive system. Therefore, it is not surprising that administration of synthetic estrogen molecules like DES to pregnant women alters the expression of Wnt [126]. The association of Hsp90 with estrogen has opposite effects on WNT-associated gene expressions. In the presence of the chaperon Hsp90, optimal activity of histone 3 lysine 4 methyltransferases, SYMD3 is observed which is required for the activation of WNT genes [126]. However, DES may deregulate
this activity by quenching Hsp90, thereby disrupting the association of Hsp90 with WNT genes [126].

The proposed model by Ruden et al. [126] attempts to explain the trans-generational effects of DES-induced uterine abnormalities. Under normal conditions, it is proposed that during development, specific methylation of CpG residues in confined regions of the chromatin in germline stem cells may occur and possibly enhancers of genes associated with uterine cancers such as c-fos are turned off [126]. However, exposure to DES may trigger hypomethylation of the enhancers of these genes allowing regulatory elements to bind to the transcriptome and induce activation of uterine cancer promoting genes [126].

Wnt signaling is also affected by DES and the expression of Wnt is crucial to maintain the methylation pattern and to replenish the uterine stem cells [126]. Since DES has opposing effects on ER-regulated genes and Wnt-mediated gene expression, the presence of this molecule can induce epigenetic effects on the genes that promote cancer phenotypes by modulating methylation patterns and histone modifications.

11.5 CONCLUSIONS
Evidence exists that dietary components affect epigenetic processes such as histone modifications [10,14–17,22,26,45,47,61,66,67,75,99,127–133]. Evidence also exists that histone modifications play a role in the development of cancer [14,74,77,134–157]. Therefore, epigenetic modulation of histone modifications by dietary components may be a useful preventive and therapeutic approach against cancer. To optimize these cancer preventive effects, it will be necessary to explore agents derived from natural sources, to
determine the conditions for their optimal effects, e.g., optimal concentration, timing, administration of the natural complex or its putative active ingredients. Mechanisms underlying the effect of dietary ingredients will also need to be elucidated. Studies discussed above suggest that epigenetic mechanisms are likely to be a major mechanism underlying the chemopreventive action of some naturally occurring agents.

Studies discussed in this review also reveal that the negative effects of some environmental pollutants may be mediated by epigenetic mechanisms. Moreover, some pollutants and the diet act synergistically via mechanisms affecting the epigenetic code [10]. Under normal conditions, epigenetic marks maintain proper gene function. However, diet and pollutants-mediated modifications of epigenetic marks affect gene expression, resulting in aberrant cellular division and triggering the neoplastic phenotype.

Elucidating the epigenetic code and mechanisms that underlie its modification by the diet and the environment may serve as a tool to predict the susceptibility of an individual to cancer and the necessary precautions or treatment required to reverse epigenetic changes. Given the diversity of histone modifications, this may not be an easy task.

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EPGENETIC APPROACHES TO CANCER THERAPY

by

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6.1 INTRODUCTION

Condensation of DNA is achieved by the interaction of basic proteins called histones that encircle 147 bp of DNA forming a structure called the nucleosome [1,2]. The histones are arranged as dimers of each subunit; H2A, H2B, H3, and H4 in the octet [2]. Histone H1 is independent of the octet but helps tether the nucleosome complex [2]. The octet complex with the DNA is so arranged that certain amino acid residues of the histones extend out serving as regulatory substrates for nucleosomal stability [1]. These substrates establish the condensed and decondensed states of the chromatin [1]. Condensation of the chromatin prevents the transcriptome machinery from binding and consequently inhibits gene expression. However, when these projected tails are modified through enzymatic transformations such as acetylation, methylation, phosphorylation, sumoylation, and ubiquitination, the accessibility of DNA changes based on the residue modified [3]. Interestingly, current research has emphasized the roles of these modifications in the transformation process of a normal cell to a tumorigenic phenotype by creating imbalances in net expression of tumor suppressor versus oncogenes or overall genomic imbalances [4]. These covalent modifications are reversible and therefore can have profound impacts on the cellular phenotype when the activities of the enzymes that mediate these modifications are altered. Intense interest has been directed toward the mechanistic pathways of these modifications in carcinogenesis. However, substrate specificity and residue-specific alterations still need to be ascertained.

In addition to histone modifications, CpG dinucleotides can be subjected to epigenetic changes by the methylation of cytosine residues [5,6]. These methylation patterns are
heritable and are governed by four isoforms of DNA methyltransferases; DNMT1, DNMT3a, DNMT3b, and DNMT3L [6]. Another area of epigenetics that still requires further exploration and can potentially compound the effects of chromatin epigenomics in a neoplastic cell is the epigenetic regulation of non-histone proteins. Epigenetic regulations of non-histone proteins can drastically affect pathways within the cell, the cell cyclical controls, and cellular phenotypes. For example, acetylation of key residues of p53 stabilizes the protein and thus the cell cyclical function with which it is associated [7,8]. This chapter discusses the current treatments that are designed to target epigenetic enzymes with the hope of reversing the epigenome of cancerous cells. Non-histone protein modifications are also important in cancerous cells and therefore the current approaches to therapy aimed at targeting non-histone proteins will also be discussed.

### 6.2 HISTONE ACETYLATION

Positively charged amino acids such as lysine (K) and arginine (R) located at amino (-NH2) terminal ends of histones are variously modified (Figure 6.1) [3]. Histones are preferentially methylated or phosphorylated at arginine residues and acetylated at lysine residues [3,9]. Acetylation of lysines initiates active gene expression. Acetylation of histone residues not only establishes euchromatin states but has crucial roles in nucleosome assembly and maintenance of chromatin states that affect various phases of the cell, including DNA repair [9-11]. Currently, no mathematical models are available that can determine the exact pattern of epigenetic marks which alter sets of genes in
FIGURE 6.1

Effects of acetylation and methylation on histone residues. The red circle represents acetyl groups, the yellow circle symbolizes methylation and the green symbolizes methylation of arginine residue. Acetylation of lysine residues is associated with gene expression whereas methylation-mediated expression is dependent on the residue methylated and the position. These reversible processes exerted by epigenetic enzymes HATs, HDACs, and HMTs largely affect genomic stabilities, local gene expression, and factors governing cell fate or phenotype. This figure is reproduced in the color plate section.
cancer tissues. Another hindrance in determining these marks is that these chemical transformations are dynamic and affect the genome globally rather than at specific targets. However, certain histone-lysine residues are specifically acetylated or deacetylated at key positions. Histone acetyltransferases (HATs) are enzymes that orchestrate the acetylation of histones and are placed in three superfamilies based on homologies to the yeast class of HATs; GNAT (Gcn5-related N-acetyltransferase), MYST (MOZ, Ybf2/Sas3, Sas2 and TIP60), and p300/CBP145 [4]. In a few cancers, dysregulation of HAT activities by mutations in the HAT genes or the dysfunction of the gene by translocations account for tumor promotion [4]. In breast cancer cells, NCOA3 (AIB1) is overexpressed and in certain leukemias, p300, CBP, and MYST3 (MOZ) translocations are observed [4]. Targeting HAT activity can affect the acetylation patterns and possibly control the expression of oncogenes that are overexpressed.

6.3 HISTONE DEACETYLASES

The dynamic equilibrium of chromatin architecture is finely regulated by the activity of HATs and HDACs. In most cancers, HATs are mutated and include chromosomal translocations of the respective HAT, but HDACs are frequently overexpressed [12]. Certain cofactors exhibit intrinsic HAT or HDAC activity and in most instances the effects are conglomerative with other complexes. Therefore aberrant recruitment of HDACs to transcription factors that affect genes such as oncogenes or tumor suppressor genes or their expression may facilitate a switch from normal to abnormal phenotype. A total of 18 HDACs have been identified and are classified into four major classes [4]. The classification of HDACs is based on the homology of the catalytic site [13]. Class IV
HDACs exhibit homologies similar to class I and II HDACs [4]. The Sir2 HDACs have prominent roles in DNA repair and different Sir2s have varied nuclear functional roles [14,15]. Chromatin organization is a well-studied area; however, the actual roles of HDACs at the gene level and their roles in specific cancers still require further elucidation. *In vitro* analysis of the effects of HDAC inhibitors has demonstrated their profound effects on inhibiting cell proliferation, and inducing cell differentiation and apoptosis [4]. HDAC inhibitors are considered important tools in cancer therapeutics and are currently being evaluated for their therapeutic efficacies *in vitro* and in clinical trials. HDAC inhibitors tested to date fall under four categories, short-chain fatty acids (SCFA), hydroxamic acid derivatives, benzamindes, and cyclic tetrapeptides [4] (Table 6.1).

<table>
<thead>
<tr>
<th>Class Type</th>
<th>Type</th>
<th>Compound</th>
<th>Class of HDAC Inhibited</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Short-chain fatty acid (SCFA)</td>
<td>Sodium butyrate</td>
<td>Class I and II</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Valproate</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Phenybutyrate</td>
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<td></td>
</tr>
<tr>
<td>II</td>
<td>Hydroxamic acid derivatives</td>
<td>Vorinostat</td>
<td>Class II</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Belinostat (FXD-101)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Panobinostat (LBH-589)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Benzamindes</td>
<td>Entinostat (SNDX-275)</td>
<td>Class I</td>
<td>[4]</td>
</tr>
<tr>
<td>IV</td>
<td>Cyclic tetrapeptides</td>
<td>Romidopar (PK-228)</td>
<td>Not determined</td>
<td>[4]</td>
</tr>
</tbody>
</table>

Inhibition of HDACs increases the acetylation levels of specific histone residues and in some instances increases stabilities of nonhistone proteins, both of which are essential to gene regulatory functions (Figure 6.2). These observations have been supported by gene expression profiling studies [16]. Of about 1750 proteins that have been identified to be acetylated at lysine residues, 200 of these become modified in the presence of HDAC inhibitors and represent a significant number that may contribute to changes in gene
FIGURE 6.2

Effects of acetylation on protein functions. Acetylation of proteins affects many different functions, some of which are listed. The double up-arrows indicate increase and the double down-arrows indicate decrease with respect to the particular function. Some of the genes affected by acetylation under specific protein functions are listed [60].
expression and probably initiate antitumor activity [16]. What needs to be determined however is the substrate specificities for each HDAC and what drugs are specific for each HDAC.

6.4 HISTONE METHYLATION AND DEMETHYLATION

Histone acetylation is key to promoting gene expression. Acetylated lysines are always associated with gene expression but their methylation status contributes to varied gene expression and primarily depends on the position and form of the methylated lysine residue (Figure 6.1). Lysines exists in mono-, di-, and tri-methylated forms and in some instances the same lysine can be acetylated or methylated, for example, K4 and K9 residues of histone H3 [17]. However, in this instance the acetylated lysine status will not govern the methylated status of the same lysine in the histone [17]. In other cases, either acetylation or methylation will influence the covalent modified status of the neighboring lysine residues and the summation of these effects will determine the outcome. A commonly found histone pattern in many cancers is the loss of H4K16 acetylation and H4K20 tri-methylation [18].

Patterns affect the histone residues globally or histones of gene-specific loci can independently influence cancer outcomes (Table 6.2). This implies that when tumor suppressor genes are down-regulated by hypermethylation, oncogenes may be stimulated by acetylation or hypomethylation. For example, hypermethylation of H3K79 promotes leukemogenesis [24]. Tumor-specific epigenetic abnormalities can stem from altered modifications of the histone residues, and/or altered expression of the enzymes that
catalyze the modifications. These changes are driven by mutations or chromosomal rearrangement of genes that code for epigenetic enzymes regardless of their epigenetic modification. As shown in Table 6.2, medulloblastoma arises from the hypomethylation of H3K9, and the loss of H3K9 methyltransferase; amplification of demethylases or acetyltransferases could trigger the outcome observed. Hyperacetylation of H3K9 could inhibit the methylation of its residue.

Like DNA, histone lysine residues are methylated by the activity of methyltransferases and utilize S-adenosyl methionine (SAM) in catalyzing the transfer of the methyl group to specific histone residues [6]. The methyltransferases are specific based on the residues they target. Protein lysine methyltransferases (PKMTs) and arginine methyltransferases (PRMTs) are specific for lysine and arginine residues respectively, and mediate mono-, di-, and trimethylation. PRMTs primarily catalyze mono- and di-methylation of histone arginine residues 2, 8, 17, and 26 of H3 and arginine residue 3 of H4 [25]. PKMTs have a conserved SET domain that is required for the methyltransferase activity and several of these have been implicated in cancers [25]. H3K27 methylation is mediated by a PKMT called EZH2 [26,27], but this enzyme is over-expressed in many tumors and appears to have major roles in cancer aggressiveness as seen in breast and prostate tissues [26,27].
In another case, leukemogenesis is promoted by the aberrant recruitment of H3K79 non-SET domain DOT1L [28]. Most of the tumor-related effects of HMTs are associated with the over-expression, amplification, and translocation of the genes coding the enzymes. Some of these include SMYD3 [29], CARM1 [30], and PRMT1 [31]. Drugs that can inhibit the activities of these enzymes are currently being investigated and a certain few are showing great promise in clinical trials. 3-Deazaneplanocin (DZNep) is a compound that targets HMTs, including EZH2, and is instrumental in inducing global hypomethylation at several lysine residues and includes H3K27 and H4K20 [32]. As seen with HDACs, in vitro analysis of DZNep treatment of tumor cell lines induced apoptosis through the activation of key target genes [32]. A combination of a HMT and HDAC inhibitors may profoundly affect the synergistic induction of apoptosis and has been demonstrated in colon cancer cells [33,34].

Like with most chemical compounds, non-specific and indirect mechanisms of action may limit their clinical applications. Certain generic compounds may inhibit HMT activity directly with low to no specificity (generic analogs of SAM such as S-adenosyl homocysteine (SAH) and sinefungin) [35]. However, more selective compounds have been identified through current screening methods. A fungal mycotoxin, chaetocin, is a potent inhibitor of H3K9 HMTases SUV39H1 and G9a (EHMT2) at IC50 concentrations of 0.8 mM and 2.5 mM, respectively [36,37]. This compound specifically depletes H3K9 di- and tri-methylation levels. However the compound exhibits cytotoxic effects independent of its inhibitory activity. Another non-SAM competitive inhibitor of HMT G9a, BIX-01294 has been effective at 1.7 mM and the inhibition is selective toward
HMTs with SET domains such as SETDB1 (ESET), and SETD7 (SET7/9) [38,39]. BIX-01294 was found after screening 12 500 compounds and is specific in action towards H3K9me2, reducing the di-methylated levels in mammalian cells. Structural analysis of the SET domain reveals that the compound binds to the H3 substrate-binding groove [38]. Both natural and chemical analogs with similar affinities to SET domains can serve as suitable inhibitors in cancer treatments. Once the mechanistic action is determined, the compounds can be modified to improve concentration efficacies and minimize non-specific or cytotoxic effects. Similar inhibitors towards PRMTs, have been found [40]. However, more robust inhibitors are yet to be discovered as the current PRMT inhibitors are non-specific and have low selectivity and activity. Pyrazole-containing CARM1 inhibitors with lower but highly potent IC50 concentrations have been reported [41]. It is encouraging that such molecules targeting essential epigenetic enzymes can potentially reverse epigenetic-mediated cancerous phenotypes and that further optimizations and discoveries of effective yet non-cytotoxic drugs need to be identified for clinical testing.

Histone demethylases (HDMs) promote hypomethylation of their target residues and the gene output is determined by the residue, or position demethylated and/or gene-specific target that is demethylated. Therapeutically, targeting HDMs can be of significant importance as oncogenes that are normally expressed through histone hypomethylation of their promoters can be switched off by hypermethylation. Certainly, smaller molecules with effective catalytic inhibitory activity toward HDMs are valuable and screening for such compounds is crucial. HDMs catalyze the removal of methyl groups from lysine residues in two ways. First by the amine oxidation reaction which is specific for mono-
and di-methylated residues and second by the hydroxylation of methylated residues creating an unstable intermediate that degrades to release formaldehyde [42]. The second process is specific towards mono-, di-, and trimethylated residues. The first process requires FAD and the second uses alpha-ketoglutarate and iron as cofactors [42]. The only known HDM to date is lysine-specific demethylase 1 (LSD1) which mediates its demethylating action through the amine oxidation process [43]. The family of Jumonji (JmjC) domain-containing proteins demethylate by the hydroxylation of the methyl groups and includes JARID1B (PLU-1) and Jumonji C (JMJC) 4 domaincontaining protein (JMJD2C) [42]. These HDMs have been implicated in tumor progression as well. LSD1 has varied roles in terms of the residues it catalyzes. H3K4 mono- or di-methylated residues are demethylated by LSD1 in conjunction with corepressor RE1-silencing transcription factor (CoREST) [44]. However, H3K9 mono- and di-methylated marks are demethylated by LSD1, in which it interacts with androgen receptor (AR) as a coactivator to enhance the demethylating function [45]. This enzyme is over-expressed in certain cancers and has been reported to be associated with aggressive prostate cancer and poorly differentiated neuroblastomas.

Since LSD1 is homologous to monoamine oxidases (MAO) [46], molecules that are effective against MAO can inhibit the activity of LSD1. One such inhibitor is the tranylcypromine. When used, this inhibitor increases the di-methylated levels of H3K4 both in vitro and in vivo and inhibits the neuroblastoma tumor growth [47]. LSD1 has been found to be inhibited by polyamine compounds, a few of which have shown remarkable positive outcomes in colon cancer cells [47]. These compounds increase the
mono- and di-methylated H3K4 levels and reexpresse many silenced genes important in colon cancer development. In breast and prostate cancer, the HMD, JARID1B, is overexpressed and demethylates H3K4me3 that induces cell proliferation, inhibits tumor suppressor functions, and results in AR coactivation [48]. JMJD2C, a H3K9me2/3 demethylase, is amplified and overexpressed in esophageal squamous carcinoma and targeting this enzyme has been proven effective in inhibiting esophageal squamous cell growth \textit{in vitro} [49]. JMJD2C in conjunction with LSD1 enhances ARdependent gene expression in prostate cancer where this enzyme is overexpressed [50]. Targeting the enzyme or cofactor can effectively inhibit the activity of HDMs such as JmjC. For example, derivatives of noggin (NOG), analog of alpha-ketoglutarate, are effective against members of the Jumonji-C family of HDMs and a dimethylester of NOG (DMOG) showing cellular activity has been reported [51]. However, these molecules that are being used as HDM inhibitors are effective at much higher concentrations. Further screening of potential drugs with higher efficacies and potencies at lower concentrations is still required and the knowledge of the structural configurations of LSD1 and JMJD2C can assist in the find.

\section*{6.5 DNA METHYLATION}

The architectural configuration of the nucleosome is strictly governed by histones and their covalent modifications. However, the DNA encompassed by the histone octet dictates cellular functions and stability. Apart from the normally associated transcription factors with promoters, methylation of CpG residues is another important mechanism regulating gene expression. Aberrant expression of this predominant epigenetic
modification has been reported to play significant roles in a variety of diseases, including cancers. Fortunately, CpG methylation can be reversed and therefore this heritable change when exposed to demethylating compounds or compounds that inhibit the catalytic function of the enzyme itself (DNMTs), presents as potential cancer therapeutic tools. DNMTs are required for CpG methylation and small molecules that can target these enzymes are being tested in vitro as well as in clinical trials. The very first DNMT inhibitor was Vidaza (5-azacytidine) and was approved for use by the US Food and Drug Administration (FDA) as treatment against myelodysplastic syndrome (MDS) [52,53]. Another molecule, Dacogen (5-aza-20-deoxycytidine, or decitabine) developed by MGI Pharma Inc. (Bloomington, MN, USA) has also been used to treat MDS [52,53]. These compounds facilitate their action through both methylation-dependent and -independent pathways and in some cases direct proteasomal degradation of the enzyme has been reported [53]. In theory, the use of these inhibitors is aimed at reversing the expression of methylation-silenced critical gene expression. Clinical trials using these molecules have shown great potential as therapeutic agents against leukemia, including MDS, acute myeloid leukemia, chronic myelogenous leukemia, and chronic myelomonocytic leukemia [53]. So far, the therapeutic improvements are seen against leukemias, although this is not the case with solid tumors since it is likely that a multitude of factors govern the growth of the mass. Cellular toxicity is also a major concern and the use of these molecules triggers cell cycle arrest by their integration into the DNA molecule itself. Therefore, it is imperative to develop or screen for drugs that have less cytotoxicity and more efficacy at lower concentrations. Another concern is the relatively low stability of these compounds in vivo and therefore modifications that enhance their stability are to
be considered when selecting the drug as a chemotherapeutic agent. It is imperative to design and develop drugs that are relatively stable, mediate the degradations of DNMTs without incorporation into DNA, and facilitate gene expression crucial to cell differentiation and apoptotic pathways.

In mammalian cells, DNMTs exist as four active forms, DNMT1, DNMT3a, DNMT3b, and DNMT3L and either singly or in combination catalyze the methylome patterning crucial to gametogenesis, embryogenesis, development, and carcinogenesis [54]. Structural analysis of these enzymes shows that the catalytic domain resides in the C-terminal region of the protein with the N-terminal essential for DNA recognition states, hemimethylated versus unmethylated. DNMT3a is ubiquitously expressed and DNMT3b is present at very low levels with the exception of a few tissues [55]. These levels change in tumor cells and global hypomethylation and regional hypermethylation of specific genes becomes an apparent pattern and has been reported to be the case in cervical, prostate, and metastatic hepatocellular carcinoma [55]. Most preclinical studies focus on the hypermethylation of key genes, in some cases tumor suppressor genes and others oncogenes and the correlation of DNMT levels, methylation patterns of the promoters, and gene expression. There appears to be some level of correlation between DNMT levels and hypermethylation; however, regression analysis does not seem to support this one-to-one correlation indicative of a much more complex regulatory mechanism in vivo [55]. Some of the commonly reported hypermethylated genes include RAR, RASSF1A, CDNK2A, CHD13, APC, p15, and p16 [56]. p15 is used as a marker to determine
leukemia transformation, and in some other tumors the levels of hypermethylation of tumor suppressor genes determine the stage of the tumor [57]. Inhibitors of DNMTs are grouped under two categories, those that interfere with the methylation of cytosine residues by chelating into the DNA complex and second, the non-nucleosides that target DNMT activity or stability (Table 6.3).

Results from both non-nucleoside and nucleoside DNMT inhibitors (DNMTi) in preclinical and clinical settings are encouraging but many of these have drawbacks that need to be revamped. Certainly the need to screen for and design small molecules with potent DNMTi activity, less cytotoxicity, and improved specificities is very evident and significant progress has been made in this direction. Combination treatments of two nucleosides and two nonnucleosides are in progress and the data generated from in vitro studies have shown remarkable synergistic DNA-hypermethylating activity. These have been well described in a review by Jiang S-W [55].

6.6 ACETYLATION OF NON-HISTONE PROTEINS
Histones are the likely targets for reversible modifications and much focus has been on understanding the roles of these modifications in cellular processes and the enzymes that catalyze these chemistries. However, gene products, primarily proteins, are in many ways subjected to similar regulations, of which acetylation and phosphorylation are key modifications. Many cellular processes are governed by the activity of proteins and are involved in cell signaling, transcription, and even protein degradation (Figure 6.2).

Reviews on the acetylation of non-histone proteins are limited, yet this protein
modification in conjunction with histone modifications is very relevant to cancer epigenomics. Acetylation of proteins can affect many aspects of protein function as shown in Figure 6.2. Especially pertinent are the effects of acetylation on p53 (tumor suppressor), nuclear factor-kB (NF-kB) (metastatic gene), and myelocytomatosis oncogene (c-Myc) (oncogene) and the plausible roles of indirect effects of HAT/HDAC inhibitors on these proteins in restoring normal cellular phenotypes.

Protein p53 is essential to many cell regulatory functions, and in particular, is important to rescue a cell from DNA damage and maintain normal cell division [58]. As seen in many cancers, p53 is mutated and the protein is dysfunctional, contributing to a tumorigenic phenotype. Activation of p53 in cells requires the phosphorylation of the protein that promotes the acetylation of key lysine residues 120, 164, 320, 370, 372, 373, 381, 382, and 386 mediated by different acetyltransferases [59,60]. Although controversial, findings strongly support that acetylation of p53 at the C-terminus end favorably enhances the DNA binding ability of the protein to its target genes [60]. K120 and K164 along with the C-terminus are required for p53 activity, and single-site mutational losses can be rescued by the acetylation of key functional residues [60]. Acetylation of p53 K120 by specific HATs, such as Tip60 and hMOF, induces genes of the apoptotic pathway [60]. When K382 is acetylated, p53 recruits CREB binding protein (CBP) that further enhances the transcription of genes, suggestive of coactivator functions [60]. In all, acetylation of p53 improves the stability and binding ability of p53, allowing for recruitment of coactivators to the transcriptional binding sites in the
promoters of genes crucial to cell cycle regulatory functions, such as for p21. Therefore, HDAC inhibitors that increase acetylated levels can contribute to a much more stable p53 even when it is mutated and can enhance its DNA-binding abilities, which otherwise would be ineffective. Further studies are necessary to validate the roles of key acetylated residues that improve the DNA-binding ability of p53 in the mutated form.

NF-κB is an important gene of the immune system and is observed to be important in the inflammatory process. This gene is also essential to cell survival, differentiation, and proliferation and is reported to be overexpressed in many tumors that are aggressive and metastatic [60]. Nuclear activation of NF-κB target genes occur only when the protein is acetylated but otherwise exists as an inactive complex tethered by Ik-B inhibitor [60]. The ubiquitination mediated degradation of Ik-B initiated by its phosphorylation, frees the NF-κB that translocates to the nucleus and binds to its target sequence. The NF-κB is
a complex of p50, p52, p65(ReLA), c-Rel, and RelB [60]. In mammals, the p50/p65 heterodimer is the most commonly found complex [60]. Modifications of these dimers are essential to many of its downstream functions. Phosphorylation of p65 initiates its acetylation at multiple sites mediated by the recruitment of p300/CBP. NF-κB target gene expression is enhanced greatly when its K221 and K310 residues are acetylated and the full activity of the protein is dependent on this modification [61]. This observation is
supported by the fact that SIRT1 deacetylation of p65 K310, HDAC1, and HDAC3
deacetylation of K221 or K310 inhibits transcription of its target genes [61]. Acetylation
of NF-κB supports many functions and the acetylation of K122 and K123 enhances the
export of the protein and re-association with IkB forming an inactive complex [62].
When p50 subunit is acetylated at positions K431, K440, and K441 the protein molecules
bind with a higher affinity to its target gene sequences [63,64]. Thus the role of
acetylation in NF-κB is many-fold. Since this gene is deregulated in many diseases
including cancers a further investigation into the application of HDACi or HATi in
regulating NF-κB functions is warranted.

Overexpression of c-Myc has been documented in many cancers. c-Myc binds and
activates target genes as a complex with Max [64]. However, regulatory functions of the
complex are solely through the transcription activation domain (TAD) of c-Myc located at
the N-terminus [64]. This domain interacts with HATs, such as GCN5 and Tip60,
forming coactivator complexes [64]. The c-Myc interacts with p300 via its TAD region
that acetylates lysines at several positions between the TAD and DNA-binding regions of
the protein, enhancing Myc turnover [64]. Thus, HAT-specific interactions with c-Myc
dictate its stability and turnover in mammalian cells, and molecules that target these
interactions by the induction of deacetylated levels are promising strategies in cancer
therapy.
6.7 FUTURE DIRECTIONS

Epigenetic phenomena affect histone and non-histone proteins and molecular compounds that target enzymes influencing these roles are important to further develop. Drawbacks of compounds such as those with high cytotoxicity, low specificity, and low stability all have to be considered when selecting an antiepigenetic compound promoting antitumor activity. One way of improving drug design or compound efficacy is by a dual approach which has been proven to be much more efficient as seen with 5 azacytidine and other chemical compounds. Further work in these areas is therefore warranted. In addition to drug-based compounds, natural plant-based products with similar characteristics need to be screened and tested.

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THE ROLE OF NUTRACEUTICALS IN CHEMOPREVENTION AND CHEMOTHERAPY AND THEIR CLINICAL OUTCOMES

by

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Abstract The genesis of cancer is often a slow process and the risk of developing cancer increases with age. Altering a diet that includes consumption of beneficial phytochemicals can influence the balance and availability of dietary chemopreventive agents. In chemopreventive approaches, foods containing chemicals that have anticancer properties can be supplemented in diets to prevent precancerous lesions from occurring. This necessitates further understanding of how phytochemicals can potently maintain healthy cells. Fortunately there is a plethora of plant-based phytochemicals although few of them are well studied in terms of their application as cancer chemopreventive and therapeutic agents. In this analysis we will examine phytochemicals that have strong chemopreventive and therapeutic properties in vitro as well as the design and modification of these bioactive compounds for preclinical and clinical applications. The increasing potential of combinational approaches using more than one bioactive dietary compound in chemoprevention or cancer therapy will also be evaluated. Many novel approaches to cancer prevention are on the horizon, several of which are showing great promise in saving lives in a cost-effective manner.

1. Introduction

The transformation of a normal cell into a cancerous phenotype requires stages of initiation, progression, and promotion by altering specific genes [1–3]. Although predisposition to cancer cannot be signaled out by a single factor, a group of factors place some individuals at a higher risk of acquiring the disease. Most of the high-risk cases may have a genetic background, but in some instances dietary choices can dictate the outcome of health. As determined by population and epidemiological studies, the predominant forms of cancer and cancer-related deaths are those of the lung and
bronchus, breast, colorectal, and prostate [4, 5]. These cancers are also more prevalent in the western parts of the world and are much lower in Asian countries. A well-balanced diet that includes more of vegetables and fruits with less fat/meat intake is in most cases a staple of many Asian countries [4, 5]. Many hypotheses have supported that diet and environment greatly influence cellular function and health [6].

Phytochemicals are plant-based chemicals that mediate their positive health benefits directly, by affecting specific molecular targets such as genes, or indirectly as stabilized conjugates affecting metabolic pathways [7]. Many genes play significant roles in the cell cycle pathway, and some of these are altered in cancer cells [1, 2]. The aim of most studies is to understand and formulate mechanistic pathways by which these naturally derived chemicals can alter the fate of a cell. For a cancerous cell to survive, it should be able to proliferate, obtain energy, and establish angiogenic pathways, in a tumor mass. Altering genes that affect these pathways can serve as suitable tools to decrease tumor mass and also allow for tumor regression. In this paper, the key focus will be on mechanistic pathways that are regulated by nutraceuticals to bring about changes in the tumor environment and serve as alternative approaches for cancer prevention and therapy (Figure 1).

The study of phytochemicals and the classification of these compounds have been previously reviewed [8]. However, in this paper only some of the most potent and promising chemopreventive and therapeutic molecules will be analyzed, with emphasis on combination therapy of these with other nutramolecules. Most phytochemicals derived
Figure 1: Cellular pathways affected by the activities of bioactive components in dietary sources. Of the natural compounds present in dietary sources, some are more involved in regulating chemopreventive pathways and some are more effective in influencing chemotherapeutic pathways. However, a few of the bioactive molecules found to date can impart both chemopreventive and therapeutic effects, such as EGCG and genistein. Compound combinations as discussed in the paper that can affect different pathways are shown and can have profound effects on tumor growth and inhibition.
from dietary sources are classified under an umbrella of specific chemical compounds as value but are germane to the function of a cell. Various studies have shown that these molecules can induce apoptosis, inhibit cellular proliferation, affect angiogenesis, and affect cancer metabolism in various cancers, all of which are hindrances to tumor growth (Figure 1) [7].

Several of the phytochemicals listed in Table 1 have been investigated in terms of their curative properties. However, one must carefully interpret the observed results in vitro and in vivo before testing the same in a clinical setting. The reasons for this are manyfold. Tests in culture are pure, in that there is only one cell type in the culture plate and all conditions are controlled, including the bioactive compound. In vivo, however, the scenario changes as there are a host of other factors that need to be taken into account, including age, weight, diet, and metabolism of the compound. A bioactive molecule in culture may be subjected to less metabolic changes and may be presented to the cell in its native form. However, in vivo the same compound may be presented differently, perhaps as a conjugate, and its mode of action may change amongst the multitude of other molecules in the host’s microenvironment. Many in vivo experiments also control for the type of diet being administered to the organism, where the concentrations or plasma availability can be adjusted. Therefore, what may work well in vitro, may have no agonistic effects or even antagonistic effects in vivo, and such discrepancies are often seen when comparing population and epidemiological studies in terms of chemical efficacy.
An effective nutraceutical is one that will have a low nontoxic dose while creating a magnitude of change in tumor dynamics. This means that at a low dose the compound should act fast on the tumor load. However, if the time taken to be effective is slow, the problems faced would be maintaining a tolerable dose and increasing bioavailability and stability. A solution to such a problem would be to use a combinatorial approach to therapy, a bioactive molecule with an effective synthetic drug or double-nutratherapy (e.g., curcumin and resveratrol). Once tumor regression sets in, dietary composition of the molecule can be adjusted.

### Table 1: Classification of nutrients as phytochemicals and their major food source availability.

<table>
<thead>
<tr>
<th>Phytochemical class</th>
<th>Bioactive compound</th>
<th>Source</th>
<th>*Molecular formula</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>Caffeine</td>
<td>Cacao, tea, coffee</td>
<td>C_{6}\text{H}<em>{10}\text{N}</em>{2}\text{O}_{2}</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td>Cacao, tea, coffee</td>
<td>C_{7}\text{H}<em>{10}\text{N}</em>{2}\text{O}_{2}</td>
<td>[20]</td>
</tr>
<tr>
<td>Monoterpenes</td>
<td>Limonene</td>
<td>Citrus oils from orange, lemon, mandarin, lime, and grapefruit</td>
<td>C_{10}\text{H}_{16}</td>
<td>[21]</td>
</tr>
<tr>
<td>Organosulfides</td>
<td>Allcin</td>
<td>Garlic</td>
<td>C_{6}\text{H}<em>{10}\text{O}</em>{2}S</td>
<td>[22--25]</td>
</tr>
<tr>
<td></td>
<td>Indole-3-carbinol</td>
<td>Cabbage</td>
<td>C_{6}\text{H}<em>{11}\text{NO}</em>{2}</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>Isothiocyanates</td>
<td>Broccoli</td>
<td>CNS</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>Stilorphane</td>
<td>Broccoli</td>
<td>C_{6}\text{H}<em>{11}\text{NO}</em>{2}S</td>
<td>[28]</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>Beta-Carotene, lycopene</td>
<td>Tomatoes</td>
<td>C_{49}\text{H}_{56}</td>
<td>[29]</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Epigallocatechin-3-gallate</td>
<td>Green tea</td>
<td>C_{28}\text{H}<em>{24}\text{O}</em>{15}</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>Black tea</td>
<td>C_{18}\text{H}<em>{14}\text{O}</em>{7}</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>Turmeric</td>
<td>C_{21}\text{H}<em>{18}\text{O}</em>{6}</td>
<td>[32]</td>
</tr>
<tr>
<td>Phenolic Acids</td>
<td>Capsaicin</td>
<td>Chilli peppers</td>
<td>C_{20}\text{H}<em>{26}\text{NO}</em>{3}</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>Ellagic acid</td>
<td>Black berries, raspberry</td>
<td>C_{23}\text{H}<em>{38}\text{O}</em>{6}</td>
<td>[34, 35]</td>
</tr>
<tr>
<td></td>
<td>Gallic acid</td>
<td>Pomegranate, nuts</td>
<td>C_{27}\text{H}<em>{32}\text{O}</em>{6}</td>
<td>[36, 37]</td>
</tr>
<tr>
<td>Stilbenes</td>
<td>Pterostilbene</td>
<td>Blueberries and grapes</td>
<td>C_{41}\text{H}<em>{40}\text{O}</em>{1}</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>Almonds, blueberries, grapes</td>
<td>C_{24}\text{H}<em>{18}\text{O}</em>{3}</td>
<td>[39]</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Daidzein</td>
<td>Soy</td>
<td>C_{41}\text{H}<em>{40}\text{O}</em>{1}</td>
<td>[9, 40]</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>Soy</td>
<td>C_{27}\text{H}<em>{20}\text{O}</em>{5}</td>
<td>[9, 40]</td>
</tr>
</tbody>
</table>

* Molecular formulas obtained through the PUBCHEM COMPOUND Database.
2. Nutraceuticals and Their Preventive and Therapeutic Roles

2.1. Genistein: A Potent Isoflavone. Many phytochemicals are currently being investigated for their promising anticarcinogenic properties. In vitro investigations have shown that some compounds exert their antitumor functions at much higher concentrations and that dietary consumption is insufficient to achieve such effective concentrations at the tumor site. Therefore, the mode of delivery is a very important factor that needs to be considered at clinical trials and during in vivo studies. The nontoxic properties of natural compounds are essential to the design of a formulated therapy. However, evidence along several lines of treatment has shown that some compounds are preferentially more potent in activity when administered early in life [9, 10]. For instance, soy-based prevention of breast cancer is thought to be more successful when soy products and their derivatives are consumed in early development [9].

Isoflavones are a group of phytochemicals that are predominant constituents of a soy-based diet [9, 10]. Among isoflavones, the three major constituents that have been shown to have remarkable influences in cancer prevention and therapy are genistein, diadzein, and glycitin [11]. They are collectively grouped as phytoestrogens for their weak estrogen-like activity and bind preferentially to ER-β receptors [12–15]. Evidence of antiproliferative activity of genistein in vitro stems from its ability to inhibit the tyrosine kinase enzyme that is most often upregulated in cancer cells [16, 17]. As a chemopreventive agent, genistein is thought to influence the differentiation process of mammary tissue. It is believed that early differentiation of mammary tissue into terminal buds, as seen in rats, serves as a chemopreventive strategy as it reduces the susceptibility of the epithelial cells in the ducts to carcinogens or estrogen and the ontogeny process.
Many aggressive cancers have altered epidermal growth factor (EGF) receptors on their cell surface allowing for a continuous downstream signaling pathway for cell division [18, 19]. This is interesting, as genistein can serve as a two-fold approach molecule for prevention and treatment. When EGF binds to its receptors, tyrosine kinase activation results in the phosphorylation of tyrosine residues of proteins involved in downstream cell signaling pathways that trigger cell division. Though studies have shown that genistein increases the EGF transcript early in development of mammary tissue, this perhaps is essential for differentiation and faster development of the breast tissue. In the long run this is a positive preventive strategy of breast lesion formation in ducts [9]. However, as seen in older rats [9], EGF mRNA decreases. Therefore, a decrease in EGF mRNA coupled with inhibition of tyrosine kinase by genistein would profoundly decrease tumor growth as cell signaling pathways are crucial to tumor maintenance.

Numerous studies have highlighted the antiproliferative role of genistein in various cancers; however, there are some studies indicating that genistein may increase cell proliferation [19, 47]. A key point to note is that nutraceuticals can be effective based on the form of genistein or its dose given at the time of the study (Tables 2 and 3), especially with respect to in vitro and in vivo models. Importantly, the downstream targets of bioactive molecules under investigation need to be ascertained for each specific tissue, if overall health applications are an issue. The nutraceutical may not affect a specific common pathway for tumors of different origins. For example, in breast tissue, EGF may be highly expressed, but, in colon cells or pancreatic cells, genes that regulate cell division other than EGF may be affected [48]. Cell culture experiments using plant-based nutrients depend on the sensitivity of the cells that are being investigated. When
cell lines are established, they are derived from cancerous tissues of specific organs and are, therefore, cell-type specific. This is drastically different in a clinical setting where the molecule has to mediate its activity amongst a host of various molecules and cell types. Therefore, the concentration of the phytonutrient in the supplemented diet will be crucial to its efficacy in the tumor environment. This can help explain the discrepancies seen in clinical trials of genistein for different tissues [47, 49, 50]. Outcomes of some in vitro studies suggest that, like other bioactive compounds, genistein appears to have a specific cut-off concentration at which this isoflavone can exhibit anticarcinogenic activity (10 μM or even higher) [48, 51], and it is, therefore, imperative to achieve such concentrations in vivo.

Isoflavones, in particular, genistein, have been extensively studied as prospective antitumor molecules in the treatment of prostate cancer [19, 52, 53]. There has been a well established line of evidence that genistein works against prostate cancer, but a majority of studies are in vitro in cultured cells [19, 52–56]. Limited clinical trials have tested the therapeutic efficacy of genistein in prostate cancer and those that have revealed inconsistencies in cell proliferation and tumor growth [57–60]. Given the inconsistencies in some of the outcomes, emphasis should be on the dose of the supplement and the form of the nutrient in the supplement at the time of administration to the patient in clinical trials. The highest achievable plasma concentration of isoflavones is 1 μM through orally administered food sources. From previous studies, this concentration is not sufficiently significant to bring about anticarcinogenic effects on the tissue. However, there is ample evidence that genistein and other isoflavones do exhibit anticancer properties and inhibit
Table 2: Pharmacokinetic studies evaluating the bioavailability of phytochemicals at given doses.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Form</th>
<th>Dose</th>
<th>Model subject</th>
<th>Experimental setup</th>
<th>Maximum plasma concentration</th>
<th>Half-life (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diadzein</td>
<td>Soy beverage</td>
<td>15 g Diadzein : genistein</td>
<td>Human postmenopausal women</td>
<td>Clinical</td>
<td>96.31 ng/mL</td>
<td>7.68</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Two soy capsules</td>
<td>(9.27 : 10.51 mg)</td>
<td></td>
<td></td>
<td>96.02 ng/mL</td>
<td>6.67</td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>Soy beverage</td>
<td>15 g Diadzein : genistein</td>
<td>Human postmenopausal women</td>
<td>Clinical</td>
<td>116.37 ng/mL</td>
<td>7.61</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Two soy capsules</td>
<td>(7.79 : 22.57 mg)</td>
<td></td>
<td></td>
<td>216.84 ng/mL</td>
<td>7.96</td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td>Curcuminoid powder form</td>
<td>10 g (n = 6)</td>
<td>Healthy human subjects (5 men and 7 women)</td>
<td>Clinical</td>
<td>2.04 ± 0.31</td>
<td>6.77 ± 0.83 for total curcumin conjugates</td>
<td>[41]</td>
</tr>
<tr>
<td>Glaucoronicide</td>
<td>curcumin (75%), demethoxy-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>curcumin (23%), and</td>
<td>12 g (n = 6)</td>
<td></td>
<td></td>
<td>1.40 ± 0.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td>Curcuminoid powder form</td>
<td>10 g (n = 6)</td>
<td>Healthy human subjects (5 men and 7 women)</td>
<td>Clinical</td>
<td>1.06 ± 0.40</td>
<td>6.77 ± 0.83 for total curcumin conjugates</td>
<td>[41]</td>
</tr>
<tr>
<td>Sulphate</td>
<td>curcumin (75%), demethoxy-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>curcumin (23%), and</td>
<td>12 g (n = 6)</td>
<td></td>
<td></td>
<td>0.87 ± 0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>Quercetin 500</td>
<td>500 mg of quercetin</td>
<td>Healthy human subjects (6 males and 4 female)</td>
<td>Clinical</td>
<td>15.4 ng/mL</td>
<td>3.47</td>
<td>[42]</td>
</tr>
<tr>
<td>Ageronone</td>
<td>Quercetin 500 plus capsule</td>
<td></td>
<td></td>
<td></td>
<td>336 ng/mL</td>
<td>Not given for plasma level, but renal clearance is 0.835</td>
<td></td>
</tr>
<tr>
<td>Resveratol</td>
<td>Uncatted immediate-release</td>
<td>500 mg resveratol/caplet</td>
<td>Healthy human subjects</td>
<td>Phase I clinical test</td>
<td>72.6 (48.9)* ng/mL</td>
<td>2.85*</td>
<td>[43, 44]</td>
</tr>
<tr>
<td></td>
<td>caplets</td>
<td>0.5 g</td>
<td></td>
<td></td>
<td>117.0 (73.1) ng/mL</td>
<td>8.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 g</td>
<td></td>
<td></td>
<td>268.0 (55.3) ng/mL</td>
<td>4.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 g</td>
<td></td>
<td></td>
<td>538.8 (72.5) ng/mL</td>
<td>8.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0 g</td>
<td></td>
<td></td>
<td>103 ± 51, nM</td>
<td>3.8 ± 0.8*</td>
<td>[45]</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>Broccoli raw</td>
<td>200 g</td>
<td>Healthy adult male subjects</td>
<td>Clinical</td>
<td>31 ± 19* nM</td>
<td>4.6 ± 0.8*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broccoli cooked</td>
<td>200 g</td>
<td></td>
<td></td>
<td>103 ± 51, nM</td>
<td>3.8 ± 0.8*</td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>Beverage 200 mL</td>
<td>112 mg</td>
<td>Healthy human subjects</td>
<td>Clinical</td>
<td>Per dose (L−1)</td>
<td>2.08–13.98 μM</td>
<td>12–24</td>
</tr>
<tr>
<td>D-Limonene</td>
<td>Oxygenated metabolite</td>
<td>30–40 ounces of lemonade</td>
<td>Healthy human subjects</td>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>perillie acid</td>
<td></td>
<td></td>
<td></td>
<td>447–596 mg D-limonene</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
cell proliferation and tumor growth. A clinical study by Gardner et al. [61] showed that treatment of patients with dietary supplements (82 mg/day aglycone equivalents) of isoflavone yielded a higher concentration of total isoflavones in the prostatic tissues than in serum. Therefore, there is a possibility of increasing the concentration of isoflavones to anticarcinogenic levels in tissue. An orally administered dose of isoflavones must withstand the rigors of the alimentary canal and become metabolized before they can be made available to tissues. Most isoflavones exist as conjugates rather than in their free state. This conjugation is perhaps the best way to present the molecule to the cell in tissues, and the hydrolysis of the conjugates in the tissue allows available free genistein delivery to the cells, as presented or tested in vitro. For pharmaceutical companies, it is required to formulate supplements with precise ratios of individual constituents of the compound. Unless a very pure form, a capsule or supplement may contain a mixture of genistein, diadzein, and glycetin (Tables 1, 2, 3, and 4). The percentage of each nutrient in the mixture will have a profound effect on the bioavailability of the compound after metabolism (Tables 2 and 3). To design such a product is certainly not easy and is dependent on many factors, but the two essential factors are the grade/stage of the tumor and the site or origin of the tissue. Of the two isoflavones, diadzein has been shown to have a lesser apoptotic effect on prostate cancer cells but can inhibit neoplastic transformation [61]. Therefore, it would be advantageous to use supplements containing the two bioactive nutrients as chemopreventive agents.
Table 3: Single-dose clinical studies evaluating the bioavailability of phytochemicals or their conjugated or active metabolites.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Route of administration</th>
<th>Form</th>
<th>Bioavailability area under the curve (AUC)</th>
<th>AUC values</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diadzein</td>
<td>Oral</td>
<td>Soy beverage</td>
<td>107 ± 49.16 ng·h/mL</td>
<td>Adjusted to the dose</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>Soy extract capsules</td>
<td>142.61 ± 43.94 ng·h/mL</td>
<td>Adjusted to the dose</td>
<td>[40]</td>
</tr>
<tr>
<td>Gelstein</td>
<td>Oral</td>
<td>Soy beverage</td>
<td>121.48 ± 70.98 ng·h/mL</td>
<td>Adjusted to the dose</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>Soy extract capsules</td>
<td>131.04 ± 60.79 ng·h/mL</td>
<td>Adjusted to the dose</td>
<td>[40]</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Oral</td>
<td>Curcuminoind powder extract capsule form (10 g)</td>
<td>35.33 ± 3.78 μg·h/mL</td>
<td>Relative</td>
<td>[41]</td>
</tr>
<tr>
<td>conjugates</td>
<td>Oral</td>
<td>Curcuminoind powder extract capsule form (12 g)</td>
<td>26.57 ± 2.97 μg·h/mL</td>
<td>Relative</td>
<td>[41]</td>
</tr>
<tr>
<td>(glucoronide + sulfate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>Oral</td>
<td>Capsule (500 mg)</td>
<td>62.5 ng·h/mL</td>
<td>Relative</td>
<td>[42]</td>
</tr>
<tr>
<td>aglycone</td>
<td>Oral</td>
<td>Capsule (500 mg)</td>
<td>2000 ng·h/mL</td>
<td>Relative</td>
<td>[42]</td>
</tr>
<tr>
<td>Quercetin- conjugated</td>
<td>Oral</td>
<td>Capsule (500 mg)</td>
<td>2000 ng·h/mL</td>
<td>Relative</td>
<td>[42]</td>
</tr>
<tr>
<td>metabolites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Resveratrol</td>
<td>Oral</td>
<td>Caplet ranging from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 g</td>
<td>223.7 ± 15 ng·h/mL</td>
<td>Relative for all</td>
<td>[43]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 g</td>
<td>544.8 (57.2) ng·h/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 g</td>
<td>78.6 (36.2) ng·h/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0 g</td>
<td>1319 (59.1) ng·h/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Stilbraphane</td>
<td>Oral</td>
<td>200 g broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raw</td>
<td>495 ± 40 nM·h</td>
<td>Relative</td>
<td>[45]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cooked</td>
<td>286 ± 139 nM·h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGCg</td>
<td>Oral</td>
<td>Average 200 mL beverage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Limonene</td>
<td>Oral</td>
<td>40 oz of Lemonade</td>
<td>5.07 to 32.59 μM·h</td>
<td>Relative</td>
<td>[62]</td>
</tr>
<tr>
<td>(perlic acid a</td>
<td>major active</td>
<td>metabolite of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>major active metabolite of</td>
<td>d-Limonene)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lycopene</td>
<td>Oral</td>
<td>Liquid form (tomato paste)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>10 mg</td>
<td>214 ± 124.8 μg·h/dL</td>
<td></td>
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<tr>
<td></td>
<td>30 mg</td>
<td>416.4 ± 183.9 μg·h/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 mg</td>
<td>421.7 ± 59.3 μg·h/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>90 mg</td>
<td>598.9 ± 396.8 μg·h/dL</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>120 mg</td>
<td>655 ± 288.6 μg·h/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* AUC value measured for resveratrol was AUC infinity with the coefficient of variance denoted in the brackets against the mean value.

**For the lowest dose of resveratrol AUC infinity value n = 1.

* AUC value measured for stilbraphane was AUC∞.  

* Based on the reference paper a list of various AUC values was given for different single doses as experimentally performed by different laboratories. Since the sample numbers were different, an average AUC value has not been given for this compound.

nd—not determined.
Of the predominant high-risk cancers, genistein appears to have a greater affect on prostate cancers [52–54]. Genistein mediates the apoptosis of cancer cells by activating and/or inhibiting genes and/or enzymes germane to tumor maintenance (Figure 1, Table 4). Some of these important mechanisms are the inhibition of the activity of tyrosine kinase, nuclear factor kappa B (NF-κB), and vitamin D 24-hydroxylase [86], activation of tumor suppressor genes, and modulation of androgen-responsive gene expression, prostate-specific antigen (PSA), and the androgen receptor (Table 4).

Of the prominent isoflavonones in soy, diadzein is less effective in its action on prostate cancer, but, unlike genistein, it is metabolized to equol, an isoflavandiol which has a longer half-life than genistein [87]. The longer half-life of equol creates the possibility of using this chemical in combination with other available nutraceuticals, where the net effect may be synergistic. However, prior preclinical tests are required to investigate this possibility.

Other dietary compounds are also of great interest in this regard. In vitro, vitamin D (Vit D) has potent tumor prevention ability and can induce differentiation and apoptosis in some of the most predominant cancers [48]. The use of nutrients as a possible treatment approach is based on the fact that chemicals occurring naturally will minimize side effects when applied to a biosystem. However, the in vitro dose at which Vit D induces its antitumor properties causes hypercalcemic conditions that can preclude treatment in patients [49]. In prostate cancer, a leading cause of cancer deaths in the western parts of the world, androgen ablation therapy is the choice of treatment.
Table 4: Assessment of the chemotherapeutic and chemopreventive effects of nutraceuticals in combination studies.

<table>
<thead>
<tr>
<th>Combination of nutraceutical</th>
<th>Dose used</th>
<th>Pathways affected or mechanistic action</th>
<th>Organ of study</th>
<th>Phase of study</th>
<th>Model of study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcumin + paclitaxel</td>
<td>50 μM/L + 10–50 μM/L based on the gene assessed</td>
<td>Inactivation of NF-κB and other metastatic genes.</td>
<td>Breast</td>
<td>In vitro</td>
<td>Human breast cancer cells MDA-MB-435 [63]</td>
<td></td>
</tr>
<tr>
<td>Carcumin + paclitaxel</td>
<td>2% w/w 10 mg/kg</td>
<td>Inhibition of metastasis</td>
<td></td>
<td>In vivo</td>
<td>Human breast cancer xenograft model</td>
<td></td>
</tr>
<tr>
<td>Carcumin + xanthorrhizol</td>
<td>Synergistic effect in the range from 5 to 20 μg/mL</td>
<td>Induction of apoptosis</td>
<td>Breast</td>
<td>In vitro</td>
<td>Human breast MDA-MB-231 cancer cells [64]</td>
<td></td>
</tr>
<tr>
<td>Carcumin + docosahexenic acid</td>
<td>Ratio of DHA to CCM MCF-7 55:30 μM MCF-10A 95:45 μM MDA-MB-35 35:35 μM SK-BR-3 60:40 μM MDA-MB 50:25 μM</td>
<td>Inhibition of proliferation, more synergistic in one of the 5 cell lines tested. Enhanced uptake of curcumin by the cells. Upregulated genes involved in cell cycle arrest, apoptosis, inhibition of metastasis, and cell adhesion. Downregulated genes involved in metastasis and invasion.</td>
<td>Breast</td>
<td>In vitro</td>
<td>Human breast cancer cells SK-BR-3, MDA-MB-231, MDA-MB-361, MCF-7, and MCF10A [65]</td>
<td></td>
</tr>
<tr>
<td>Carcumin + genistein</td>
<td>10 μM + 25 μM 10 μM + 25 μM 11 μM + 25 μM</td>
<td>Change in cell morphology and growth inhibition</td>
<td>Breast</td>
<td>In vitro</td>
<td>T47D and MCF-7 MDA-MB-231 [66]</td>
<td></td>
</tr>
<tr>
<td>Carcumin + sulphinosine</td>
<td>15 μM + 10 μM</td>
<td>Alter multidrug resistance genes. Alters the cell cycle with cells inhibited primarily in the S G2/M phase of the cycle</td>
<td>Lung</td>
<td>In vitro</td>
<td>NCI-H460/R [67]</td>
<td></td>
</tr>
<tr>
<td>Carcumin + celecoxib</td>
<td>10–15 μM/L + 5 μM/L</td>
<td>Inhibition of cell proliferation and induction of apoptosis.</td>
<td>Colon</td>
<td>In vitro</td>
<td>IEC-18-K-ras (Cox-2, high levels) Caco-2 (Cox-2, low levels), and SW-480 (no COX-2) [68]</td>
<td></td>
</tr>
<tr>
<td>Collect + 5-amino salicylic acid (ASA)</td>
<td>Collect only 20 μM</td>
<td>Inhibition of tumor growth by induction of apoptosis.</td>
<td>Colon</td>
<td>In vitro</td>
<td>HT-29 cells</td>
<td></td>
</tr>
<tr>
<td>Collect + 5-amino salicylic acid (ASA)</td>
<td>150 mg/kg + 50 mg/kg</td>
<td>Inhibits abnormal crypt formation</td>
<td>Colon</td>
<td>In vivo</td>
<td>Chemical induction of tumors by 1,2-dimethyl hydrazine (DMH) model in rats. [69]</td>
<td></td>
</tr>
<tr>
<td>Combination of nutritional</td>
<td>Dose used</td>
<td>Pathways affected or mechanistic action</td>
<td>Organ of study</td>
<td>Phase of study</td>
<td>Model of study</td>
<td>Reference</td>
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<tr>
<td>Curcumin + PEITC</td>
<td>25 μM + 10 μM</td>
<td>Additive effect in the induction of apoptosis.</td>
<td>Prostate</td>
<td>In vitro</td>
<td>PC-3 C4 cell line</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>3 μM + 2.5 μM</td>
<td>Inhibition of tumor growth through inhibition of Akt and NF-κB pathways.</td>
<td></td>
<td>In vivo</td>
<td>NCI-Immunodeficient (nu/nu) mice bearing s.c. xenografts of PC-3 human prostate cancer cells</td>
<td>[70]</td>
</tr>
<tr>
<td>Pure 3—curcumin + resveratrol + EGCG; Pure 4—apigenin + baicalein + genistein + quercetin; Pure 7—Pure 3 + Pure 4; Crude 7—soy + sencha leaves + turmeric + yucca roots + saw palmetto + chamomile flowers + ginkgo</td>
<td>Individual compounds, Percentage composition in the diet not defined</td>
<td>Inhibit growth by inhibiting hedgehog signaling pathways.</td>
<td>Prostate</td>
<td>In vitro</td>
<td>PC-3, LNCaP and mouse cell line TRAMP-C2</td>
<td>[71]</td>
</tr>
<tr>
<td>D-Limonene + docetaxel</td>
<td>0.2 mM + 1.9 nM</td>
<td>Induction of apoptosis by the regulation of proteins involved in mitochondrial apoptotic pathways</td>
<td>Prostate</td>
<td>In vitro</td>
<td>Dunning R3327-H prostate adenocarcinoma model</td>
<td>[72]</td>
</tr>
<tr>
<td>Tomato powder + broccoli powder (10:1) g/100 g of diet</td>
<td>11 nM of lycopene per g of diet and broccoli powder, 1.6 μM of gholcarpratin, 5.9 μM of gholcbrascin, 3.9 μM of gholcmasturtin, and 2.1 μM of gholcbrobrassin,</td>
<td>Reduction of tumor growth mediated by reduced cell proliferation and induction of apoptosis</td>
<td>Prostate</td>
<td>In vivo</td>
<td>Human prostate carcinoma DU-145 and normal prostate epithelial FZ-HPV-7 cells</td>
<td>[73]</td>
</tr>
<tr>
<td>Lycopene + ketosamine (fructose/amino acid Fru/His)</td>
<td>1 μM/L + 2 mM/L</td>
<td>Synergistic effect in inhibiting cell proliferation mediated processes. Antioxidant activity to prevent initiation of tumors.</td>
<td>Prostate</td>
<td>In vitro</td>
<td>Mat-LyLu rat cells</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td>20 μM/L + 5.6 mM/L</td>
<td>Reduce tumor growth and volume.</td>
<td></td>
<td>In vivo</td>
<td>Subcutaneous injections of Mat-LyLu cells in male Copenhagen rats</td>
<td></td>
</tr>
<tr>
<td><strong>Combination of nutraceuticals</strong></td>
<td><strong>Dose used</strong></td>
<td><strong>Pathways affected or mechanistic action</strong></td>
<td><strong>Organ of study</strong></td>
<td><strong>Phase of study</strong></td>
<td><strong>Model of study</strong></td>
<td><strong>Reference</strong></td>
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</tr>
<tr>
<td>Lycopene + docetaxel</td>
<td>1 μM + 1 nM</td>
<td>Synergistically enhances the antiproliferative effects of docetaxel.</td>
<td>Prostate</td>
<td>In vitro</td>
<td>Human PC-3, LnCaP, DU145 cells</td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg lycopene + 10 mg/kg docetaxel</td>
<td>Reduced tumor volume and growth by affecting the levels of IG-FR receptor that is highly expressed in a majority of prostate tumors. Inhibited Akt signaling and suppressed surviving necessary for tumor growth.</td>
<td>Prostate</td>
<td>In vivo</td>
<td>Xenograft of DU145 cells in NCR-nu/nu (nude) mice</td>
<td></td>
</tr>
<tr>
<td>Quercetin and a pH-modified citrus pectin (MCP)</td>
<td>1.6 mg/mL + 1.6 mg/mL</td>
<td>Inhibits the self renewal capacity of prostate cancer stem cells (PCSCs) by synergistically inducing apoptosis, decreasing cell viability in spheroids, cell migration, invasion and colony formation.</td>
<td>Colon</td>
<td>In vivo</td>
<td>Balb/c mice</td>
<td>[76]</td>
</tr>
<tr>
<td>Quercetin + EGCG</td>
<td>20 μM + 0–60 μM</td>
<td>Reduction in the growth of solid primary tumors.</td>
<td>Prostate</td>
<td>In vitro</td>
<td>Prostate cancer stem cells (PCSCs)</td>
<td>[31]</td>
</tr>
<tr>
<td>Resveratrol + estrogen (E2)</td>
<td>10 μM + 1 nM</td>
<td>Antagonistic estrogenic effects in suppression of progesterone receptor.</td>
<td>Breast</td>
<td>In vitro</td>
<td>Human MCF-7 cells</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>Either all at 0.5 μM and 5 μM, or 20 μM</td>
<td>Synergistically inhibited cell proliferation and induced apoptosis.</td>
<td>Breast</td>
<td>In vitro</td>
<td>Human MDA-MB-231 cells</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>0.5, 5, and 25 mg/kg body weight in a 100-μL volume</td>
<td>Inhibited cell cycle progression with predominant cell cycle arrest in the G2 phase.</td>
<td>Breast</td>
<td>In vivo</td>
<td>Breast cancer xenografts in mouse models</td>
<td></td>
</tr>
<tr>
<td>Resveratrol + quercetin + catechin</td>
<td>50 μM + 5 mM</td>
<td>Reduced primary tumor growth and, therefore, inhibit tumor progression.</td>
<td>Breast</td>
<td>In vitro</td>
<td>MCF-7</td>
<td>[78]</td>
</tr>
<tr>
<td>Combination of Nutraceuticals</td>
<td>Dose used</td>
<td>Pathways affected or Mechanistic action</td>
<td>Organ of Study</td>
<td>Phase of Study</td>
<td>Model of Study</td>
<td>Reference</td>
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</tr>
<tr>
<td>Resveratrol + α-Lipoic acid</td>
<td>50 µM + 2 mM/L</td>
<td>Inhibited cell proliferation and induced differentiation. Attenuated p27 (Kip1) levels but enhanced p21 (Waf1/Cip1) expression.</td>
<td>Colon</td>
<td><em>In vitro</em></td>
<td>Caco-2</td>
<td>[79]</td>
</tr>
<tr>
<td>Resveratrol + 5-Fluorouracil</td>
<td>200 µM + IC_{50} 800 µM</td>
<td>Inhibited cell proliferation and induced apoptosis by increase in caspase 6 activity</td>
<td>Colon</td>
<td><em>In vitro</em></td>
<td>HCT116 p53+/+ and p53−/−</td>
<td>[80]</td>
</tr>
<tr>
<td>Resveratrol + genistein</td>
<td>250 mg/kg each in the AIN-76 diet</td>
<td>Suppressed prostate cancer development and mediated apoptosis by affecting the expression of steroid-receptor coactivator-3 and insulin-like growth factor-1</td>
<td>Prostate</td>
<td><em>In vivo</em></td>
<td>Simian Virus-40 T-antigen-(SV-40 Tag-) targeted probasin promoter rat model, a transgenic model of spontaneously developing prostate cancer.</td>
<td>[81]</td>
</tr>
<tr>
<td>Genistein + sulforaphane</td>
<td>5 µM/L + 15 µM/L</td>
<td>Affected DNA methyltransferase activity and reversed the gene expression of promoter hypermethylated genes of retinoic acid receptor h (RARβ), RARB, p16INK4a, p16 and O6-methylguanine methyltransferase enhanced growth inhibitory effects.</td>
<td>Esophagus</td>
<td><em>In vitro</em></td>
<td>KYSE 510 cells</td>
<td>[82]</td>
</tr>
<tr>
<td>Sulforaphane + benzylisothiocyanate</td>
<td>10 µM + 10 µM</td>
<td>Changed cell morphology and inhibited cell proliferation. Reduced cell viability that correlated with reduced pSTAT3 levels and an increase in PARP Cleavage</td>
<td>Pancreas</td>
<td><em>In vitro</em></td>
<td>PANC-1 cells</td>
<td>[83]</td>
</tr>
<tr>
<td>Sulforaphane + apigenin</td>
<td>10 µM + 10 µM</td>
<td>Synergistically induced phase II enzyme UDP-glucuronyl transferases (UGT1A1) transcript but to a lesser effect the protein level. Mediates this action by the induction of NFκB</td>
<td>Colon</td>
<td><em>In vitro</em></td>
<td>CaCo-2</td>
<td>[84]</td>
</tr>
</tbody>
</table>
However, as the cancer becomes aggressive, hormone ablation therapy fails, and progression ensues via androgen-independent pathways. Therefore, alternate therapies are very much in demand. Vitamin D is an alternate form of treatment in prostate cancer (PCA) and is shown to induce apoptosis in PCA cells in vitro. However, all PCA cell lines in vitro are not equally receptive to the vitamin D treatment or genistein [88]. Cell lines such as DU145 prostate cancer cells are especially more resistant as they express high levels of CYP24, an enzyme that catabolizes Vit D3 into less active metabolites [88]. To circumvent this problem, a recent study showed that a dual combination therapy, of DU145 to genistein and Vit D3, increased the sensitivity of the cells to Vit D3 by decreasing CYP24 expression. What is interesting to note is that the combination approach not only lowered the effective dose, but was able to abrogate cell proliferation as well. This lowered concentration of genistein at 100 nM is achievable in vivo through dietary sources, and clinical studies would be required to determine the localization of genistein and Vit D3 in prostatic tissues.

An in vivo study for colorectal cancer has demonstrated a similar effect [89], but in this case the mice were given a single gavage of 250 μg of genistein. This mode of nutrient administration is useful for a preclinical test and probably has applications as a chemopreventive supplement. However, in terms of a clinical setting, patients are often
exposed to a host of other nutrients or isoflavones in their diet, and; therefore, an *in vivo* model replicating such an environment with various percentages of isoflavones will allow for a better understanding of concentration and bioavailability of genistein that can mediate an apoptotic effect and reduce CYP24 expression in colonic tissues in the presence of vitamin D.

The antimetastatic properties of genistein are mediated by altering the expression of NF-κB, and inhibiting the tyrosine kinase enzyme [17, 90]. Non-small-cell lung cancer (NSCLC) is a highly aggressive form of lung cancer with a poor prognosis. Therefore, alternate approaches that drastically reduce tumor growth are of utmost importance. Activation of epidermal growth factor receptor tyrosine kinase (EGFR-TK) enhances the cell signaling pathways allowing tumor growth. The use of drugs that inhibit EGFR-TK and affect NF-κB, a gene whose transcribed products are essential for invasion and metastasis, can induce a more aggressive approach of reducing tumor size and the spread of the disease. A clinical therapy should be aimed at reducing tumor growth and spread by inhibiting mechanisms that contribute to the activation of metastasis. In NSCLC, genistein remarkably enhances the effects of EGFR-TK inhibitors, such as erlotinib and gefitinib, when used in combination with each of them, respectively. This effect was seen to be mediated by a marked reduction in NF-κB and others, such as EGFR, pAkt, COX-2, and PGE(2), essential for regulating genes that control division, proliferation and metastasis [90]. A few studies have shown how a combined approach can lower the effective dose concentration even of chemotherapeutic drugs, minimizing potential side effects. A study conducted on breast and pancreatic cells showed that, when the cells were primed with genistein, lower concentrations of the chemotherapeutic drugs were
needed to significantly bring about growth inhibition and apoptosis than with the drugs alone. In addition, NF-κB was transcriptionally inhibited in the combined treatment [90].

From a number of investigations, a common thread of evidence seems to emerge that considerable variation in the efficacy of bionutrients in cancer treatment exists and differs even among the same cell lines tested. The reasons for this are manyfold (Table 5). Cell lines derived from the same tissue hypothetically should be sensitive to the same dose or chemical class of the phytonutrients, but such is not always the case. Alternate medicinal approaches have an important task to identify crucial factors that change the sensitivity of the chemical and determine chemical modifications that would be necessary to modulate more synchronized results across several cell lines expressing similar genotypic and phenotypic signatures.

Table 5: Factors conducive to the anticarcinogenic efficacy of nutraceuticals

<table>
<thead>
<tr>
<th>Factors</th>
<th>Possible effects on the bioactive components in the dietary supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioavailability</td>
<td>Metabolism</td>
</tr>
<tr>
<td></td>
<td>Time taken to achieve maximum plasma concentration</td>
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<tr>
<td></td>
<td>Maximum plasma concentration, half-life</td>
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<tr>
<td>Method of ingestion</td>
<td>Oral</td>
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<td></td>
<td>Intraperitoneal</td>
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<td></td>
<td>Subcutaneous</td>
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<tr>
<td>Form of ingestion</td>
<td>Powder/capsule</td>
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<tr>
<td></td>
<td>Liquid</td>
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<td></td>
<td>Cooked (solid)</td>
</tr>
<tr>
<td></td>
<td>Raw (solid)</td>
</tr>
<tr>
<td>Formulation</td>
<td>Ratio of pure to the compound conjugates</td>
</tr>
<tr>
<td>Stability</td>
<td>Preference for an acidic or basic environment (pH)</td>
</tr>
<tr>
<td>Mechanism of action</td>
<td>Direct via receptors on the cell surface or into the nuclear region via channels</td>
</tr>
<tr>
<td></td>
<td>Indirect conjugated metabolites</td>
</tr>
<tr>
<td></td>
<td>Affecting parts of metabolic pathways</td>
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</tbody>
</table>
2.2. *Epigallocatechin-3-gallate (EGCG): A Potent Flavanol.* Of the major food-derived phytochemical constituents that are extensively studied for their chemopreventive and chemotherapeutic use, EGCG and genistein are by far the most investigated. EGCG has been shown to have numerous anticancer properties which include antiangiogenic activity by affecting the transcriptional expression of vascular endothelial growth factor (VEGF) [91], inhibiting tumor initiation and promotion by inhibiting signal transduction pathways via [phosphatidyloinositol 3-kinase-Akt kinase- NF-κB] [92–94], inhibiting EGFR [95], inhibiting Her-2 receptor phosphorylation in breast carcinoma cells that constitutively expresses Her-2/neu receptor [95], inducing apoptosis in estrogen receptor-(ER-) independent breast cancer cells [96], causing antimetastatic activity [97], inhibiting proteasome formation [98], inhibiting glucose-regulated protein (GRP78) activity [99]; inhibiting insulin-like growth factor-I receptor (IGF-IR) [100], and preventing invasion of tumors by inducing HMG-box transcription factor 1 (HBP1) transcriptional repressor, an inhibitor of the Wnt signaling pathway crucial for tumor-invasive property [101].

The serum level concentrations of EGCG are important to ensure that an effective response is seen without adverse or even tumor-promoting functions. Studies have shown that high doses of catechins that include a higher concentration of two prominent compounds, epicatechin gallate (ECG) and EGCG, induce hypoxia-inducible factor 1 which is responsible for activating genes related to hypoxia conditions. This allows tumor cell proliferation through alternate survival pathway mechanisms [102]. Most breast cancers are ER dependent; however, for breast cancers and others that are ER independent, EGCG inhibits the growth of tumor cells through the process of apoptosis [96, 103]. As seen in MDA-MB-468 ER-negative cells, cellular apoptosis is mediated by
inducing p53 and Bax proteins that enhance apoptotic functions in cells [96]. Such observations have been corroborated by in vivo studies using animal models [97].

Most studies have shown that anticancer properties of EGCG are mediated at higher doses. However, such doses may be irrelevant to clinical applications as they may be physiologically unachievable through dietary consumption. Therefore, clinical trials should be aimed at achieving desired anticancer preventive or tumor functions at much lowered doses. Such outcomes are possible with a dual-drug approach. One study [95] demonstrated that combining EGCG with the drug taxol, which is commonly used to treat breast carcinomas, lowered the effective dose of EGCG, ranging from 0.1–1.0 μg/mL which is a serum obtainable level through metabolism. This same group showed that higher doses (30–40 μg) of EGCG were required to mediate a similar effect when used alone [95].

EGCG can be exploited as a chemopreventive agent if it prevents cancerous lesions from occurring at lower dose concentrations and for prolonged periods of time. Most in vitro studies have used relatively high doses of EGCG and such doses may prove to be more tumor promotive than preventive in longer exposure time periods. In a study designed by Pianetti et al. [92], contradictory results on the effects of EGCG on Her-2/neu overexpressed receptor in NF639 breast cancer cells was observed. At short exposure times, EGCG was very effective in reducing cell proliferation, but at prolonged exposure cells became resistant to EGCG with increased levels of NF-κB. This observed change in drug-induced resistance was related to the activation of mitogen-activated protein kinase. It appears that single doses or one specific chemical constituent is mostly insufficient to induce tumor suppression or regression. Such in vitro data outcomes
emphasize that a dual-drug treatment approach is necessary to treat the disease. This also signals that the timing of the nutradrug that is administered is important. Perhaps EGCG should be administered early in treatment, but later other phytochemicals or drugs, in conjunction with EGCG, may need to be administered in the treatment regimen. In their dual-drug treatment of NF639 Her-2/neu breast cancer cells, Yang et al. found that treating the cells initially with EGCG lowered cell proliferation and the later introduction of the MAPK inhibitor, U0126, reduced invasive phenotype [93].

Most studies determining the anticancer drug properties of EGCG are preclinical. For better understanding of specific EGCG effects, clinical trials should be carefully designed to include parameters that influence EGCG effectiveness. EGCG has different roles in ER-dependent versus ER-independent receptors, and, therefore, the type of diet needed to emulate in vitro doses need to be clearly understood through clinical trials and careful pharmacokinetic studies of these doses in healthy individuals, ER-positive breast cancer patients, and ER-independent tumors.

In testing phytochemicals of the same or different class it is rather uncertain which markers are necessary to determine comparable dosage values for in vitro versus in vivo efficacies. Formulation of a diet is one of the major deciding factors in the functional efficacy of a chemical constituent. It defines the concentration of the dose that will be available in vivo, after metabolism, and determines the diet that needs to be given to achieve such an outcome. Even though single-dose individual or mixed phytochemical treatments are currently available to cancer patients, they are relatively new and much more research in this direction is warranted. One such therapy that is rapidly gaining
importance and holds promise for future cancer treatments is combination therapies using plant-based chemical compounds known as nutraceuticals.

3. Combinatorial Therapy: A Promise of the Future (See Table 3)

In prevention or treatment, combinatorial approaches can be of the following types: a phytonutrient and an effective drug, two or more phytonutrients, a synthetic phytonutrient and an effective drug, or a synthetic phytonutrient and a natural nutrient. Studies in the last few decades have focused attention on unraveling the protective properties and mechanistic actions of many phytochemicals. Still the pharmacokinetics of quite a few of these phytochemicals are not known, and, for a few that are known, there is much variability based on mode and form of delivery, dose, and the model organism of study (Tables 2, 3, and 4). Another interesting approach to enhancing curative and preventive properties of these nutrients is combination therapies. The therapy is based on the factual information available at hand and using the potent properties of one with that of another to enhance synergistic or additive actions (Figure 1). In this paper, groups that have worked with different phytomolecules belonging to a different or the same chemical class of compounds have been analyzed for their antitumorigenic activities, and the overall results of the experiments for each group are described in Table 4.

3.1. Curcumin and Taxol (See [63]). Primary breast cancer cells are commonly treated with the drug taxol. Sustained chemotherapeutic treatment with this drug has often resulted in drug resistance and tumor progression. Many chemotherapeutic drugs induce the expression of the metastatic gene NF-κB which encourages tumor progression.
Interestingly, natural-based compounds that are pharmacologically safe have been shown to inactivate NF-κB expression. Taxol is a powerful drug in the treatment of cancer therefore, in order to prevent metastasis, a combination of Taxol with curcumin has been shown to downregulate the expression of NF-κB and induce apoptosis.

3.2. Curcumin and Xanthorrhizol (See [64]). A study conducted on an invasive breast tumor cell line, MDA-MB-231, has shown how and when compounds added to the cells determine the overall efficacy of the treatment. A sequential addition of curcumin and xanthorrhizol (a rhizomal sesquiterpenoid of Curcuma xanthorrhiza) in culture resulted in additive and antagonistic effects depending on which compound was added first to the culture. However, simultaneous addition of the compounds resulted in synergistic effects at lower concentrations and agonistic effects at higher concentrations. Such experiments provide evidence that the efficacy of a drug is dependent on dose, time, and how it is presented to the cells. Therefore, results obtained might be contradictory if doses used are simply antagonist or additive. For a successful combination therapy or prevention, synergistic doses are more relevant to mediate downstream effects, as lower concentrations of the test biomolecules will be required.

3.3. Curcumin and Docosahexaenoic Acid (DHA) (See [65]). DHA is a dietary compound present in fish oil that has been shown to have potent chemopreventive affects against cancer. Chemotherapeutic effects of compounds are often analyzed using in vitro models. However, what is most often observed is that all cells from the same tissue sample do not react the same way to the test compound. It is essential to have a
chemopreventive or therapeutic agent that can induce its effects on a wide range of
cancerous cells arising from the same tissue. In this study, the authors analyzed five cell
lines expressing different cell surface receptors (Table 6) which make them susceptible to
chemotherapeutic compounds but in different ways and to different degrees. The
combinatorial synergistic doses for each cell line were different, as shown in the Table 4.
In particular, one breast cancer cell line, SK-BR-3, which is ER-negative exhibited a
higher uptake of curcumin in the presence of DHA. DHA does not directly contribute to
cell inhibition, but the combination of this compound with curcumin greatly enhances the
uptake of curcumin by the cells. This compound, DHA, can reach a plasma concentration
level of 200 μM. Although the focus of this study was entirely based on the SK-BR-3 cell
line, the effects of reduced synergy on other cell lines in terms of transcriptome effects
need to be investigated. Mammary tumors may contain a heterogenous population of
cells exhibiting different surface receptors. Using combination therapy should be aimed
at reducing the populations of all these cell types within the tumor site to truly exhibit
antitumor potency with minimal side effects.

Table 6: Surface receptors expressed by breast cancer cells that alter their sensitivity to
treatment.

<table>
<thead>
<tr>
<th>Breast cancer cell line</th>
<th>Receptors on the surface</th>
<th>Cancer type</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-BR-3</td>
<td>Negative</td>
<td>Positive</td>
<td>Invasive</td>
<td>[65]</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Negative</td>
<td>negative Adenocarcinoma</td>
<td>Invasive</td>
<td>[65]</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>Positive Positive</td>
<td>Positive</td>
<td>Metastasis</td>
<td>[65]</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Positive Isoform B of PR</td>
<td>Negative</td>
<td>Invasive</td>
<td>[65]</td>
</tr>
<tr>
<td>MCF10AT</td>
<td>Positive Variable</td>
<td></td>
<td>Premalignant</td>
<td>[65]</td>
</tr>
</tbody>
</table>
3.4. *Curcumin and Genistein (See [66]): A Preventive Strategy.*

The aim to use natural compounds in diets is to render the chemopreventive properties of the compounds to the tissues. Numerous studies have shown that single dosage of compounds used alone is effective for chemoprevention. The problem faced is the inability to achieve high serum concentrations *in vivo*. Although combination studies are just beginning to surface as more prominent approaches in clinical treatment, studies, though limited, have shown that synergistic effects of the compounds are able to be achieved at much lower doses than when compounds are used alone. Especially in cancers that are hormonally regulated, the tissues are often exposed to external or internal hormonal stimulation, like estrogen, as in the case of breast tissue. Environmental agents that mimic estrogen-like activity can often stimulate or initiate the carcinogenic process. Curcumin, a curcuminoid, and genistein, an isoflavone, are derived from two different chemical classes, yet they have been known to inhibit a variety of tumor types *in vitro* and *in vivo*. Clinical trials of these compounds individually have been tested [19, 60, 104, 105]. The mechanistic action of the individual compounds in many different cancers has been investigated as well. However, using these compounds in combination drastically affects the development of tumors by mediating changes in shape and growth inhibition. Such changes were observed both in ER-positive and ER-negative cells, indicative of the dual use of such a combination in prevention and therapy.

3.5. *Curcumin and Sulfinosine (SF) (See [67]).* The ineffectiveness of certain drugs in prolonged chemotherapy stems from the resistance that some cancers develop with time. This is one of the major obstacles in cancer therapy, especially in cancers that are
multidrug resistant (MDR). The problem in finding a suitable cure for non-small-cell lung cancers is the MDR phenotype it exhibits. Treating MDR cells such as non-small-cell lung carcinoma NCI-H460/R cells with a commonly employed drug, SF (obtained by the amination and subsequent oxidation of 6-thioguanosine), in cell cultures has been shown to inhibit cell growth. This observed cytotoxicity is enhanced several folds when low doses of the natural compound, curcumin, are used in combination, which are otherwise ineffective unless very high concentrations are used. These compounds mediate a synergistic effect in regulating the cell cycle phases and downregulate MDR genes, thereby, enhancing tumor regression phenotypes even in the presence of mutated p53 molecules.

3.6. Curcumin and Celecoxib (See [68]) Cyclooxygenase-2 (COX-2) expression is central to the carcinogenesis of colorectal cancers. Compounds that regulate the expression or activity of COX-2 in cells may be instrumental in mediating chemotherapeutic effects on the tissue or cells. Celecoxib is a potent inhibitor of COX-2 and is presumed to target its active site. However, prolonged exposure to celecoxib results in cardiovascular problems. It appears that monotherapy regimes are very effective in inhibiting cancer growth, proliferation, metastasis, and invasion, as seen in numerous in vitro and in vivo models. However, prolonged exposures at concentrations relatively higher than what can be achieved with combination doses may result in unwanted side effects. Testing the efficacy of celecoxib with cucumin showed that at lower doses of celecoxib it was possible to enforce synergistic inhibitory growth effects on colon cells which expressed various levels of COX-2. Like many other in vitro investigations, this study emphasizes
the fact that combining powerful drugs with naturally available potent compounds can reduce the dose needed to mediate potent anticarcinogenic effects with minimal side effects. Clinically, such studies are relevant as the doses used or needed are within the physiologically dose range. With colon cancer having such a high incidence rate in the western populations, such therapies can be taken as advantage, and biomolecules having preventive potential against the formation of precancerous lesions need to be supplemented in diets of patients at high-risk.

3.7. Collect and 5-Aminosalicylic Acid (5-ASA) (See [69]). Collect is a novel chemotherapeutic dietary drug with a formulation of curcumin, a turmeric extract (95% curcuminoids) mixed with turmeric powder 1 : 1, green tea (60% polyphenols and 25% EGCG) in a 2 : 1 ratio, and 0.1 mg/mL of L-selenomethionine. 5-ASA is an anti-inflammatory drug, which has been shown to have a preventive role in polyp formation that is thought to occur via the inflammation process in conditions like inflammatory bowel disorder. Collect has been effective against HT-29 human colon adenocarcinoma grade II cells in vitro, and this nutraceutical complex in combination with 5-ASA has been shown to inhibit the formation or growth of chemically induced aberrant crypt foci (ACF) in rat models. The molecular mechanism by which this inhibition is mediated is via the inhibition of COX-2 pathways in HT-29 cells, which has been supported by in vitro studies of other groups [106, 107]. However, growth inhibition can be affected via COX-2-independent pathways possibly through mechanisms that are regulated by the functional polyphenol complex in coltect. Such complex mixtures are of clinical significance as many different control mechanisms can be regulated by the presence of
individual constituents of the polyphenols which are a part of the formulated mixture of colletct.

3.8. Phenylethylisothiocynate (PEITC) and Curcumin (See [27]). Most prostate cancers begin as a hormone-dependent tumor, and the hormone is primarily androgen. However, the more aggressive forms of prostate cancer are androgen-independent and hormonal therapies fail to be effective. Alternate therapies are, therefore, necessary to treat such aggressive forms. Most cancerous cells express various surface receptors that propagate cellular growth. Targeting such receptors can be an effective chemotherapeutic approach. Curcumin, obtained from Curcumin longa, has been shown to inhibit the phosphorylation of EGFR, inhibit the Akt signaling pathway, and negatively regulate NF-κB. It is an effective molecule against prostate cancer. Phenylethylisothiocyanate, a phytochemical in cruciferous vegetables, has been shown to inhibit prostate cancer cell growth in vitro and this observation has been supported by epidemiological studies showing that consumption of cruciferous vegetables has an inverse effect on prostate cancer risk. When two bioactive molecules with similar effects are used in treating hormonally independent tumors in affecting receptor mediated signaling, the effects are more pronounced than when used as individual compounds. With PEITC and curcumin, the observed effect was more additive than synergistic, but cell growth inhibition was profoundly affected by the inhibition of NF-κB pathways and Akt signaling pathways. Such responses were seen at lower physiological achievable doses. These results were corroborated by in vivo studies in mice using human PC-3 prostate cancer cells [70].
Since EGCG has similar effects on prostate cancer cells, EGCG could also possibly serve as a substitute in place of curcumin for such a treatment strategy.

4. D-Limonene and Its Combination Therapies (See Table 3)

Although a few studies have shown that D-limonene, an abundant monoterpene in citrus oils, exhibits antimitogenic activity, its alcohol-derivated perillyl alchohol (PA) has a greater inhibitory effect on cell migration in cancerous cells [108]. A study by Reddy et al. [108] used subtoxic doses of PA to determine this effect. Further preclinical studies are necessary to determine the effective yet nontoxic serum/tissue concentration that can be achieved from a diet rich in citrus intake, in conjunction with phytonutrients of the same class or a different class. Not much is known about the percentage composition of D-limonene and its metabolized constituents that are required to achieve an effective monterpene anticarcinogenic activity. In comparison to its oxygenated derivatives, limonene has the least cytotoxic effect on both noncancerous and cancerous breast cell lines and, therefore, can be applicable in chemoprevention [109]. D-limonene appears to be more effective against chemically induced colonic crypt foci [110]. These foci are preneoplastic lesions and are biomarkers for the progression into colon cancer. In colonic crypts that are chemically induced, limonene asserts its effect by inhibiting the activity of ornithine decarboxylase, an enzyme essential for the polyamine biosynthesis pathway. This pathway regulates the cell cycle, and D-limonene-dependent inhibition of ornithine decarboxylase (ODC) encourages an antiproliferative activity. If aberrant crypt foci are the initial markers for colon carcinogenesis, and D-Limonene and its derivatives assert their roles against initiation and promotion phases of cancer, then a diet rich in citrus
foods can prevent crypt formation. Therefore, D-limonene appears to have potential as a chemopreventive agent in colon carcinogenesis. However, in vivo studies often do not correlate with results in vitro for many of the reasons discussed earlier. Once the intake of a compound is deemed safe for human consumption, it is imperative to analyze and study the mechanistic and metabolic functions in human subjects to determine the efficacy of the nutrient in question. As in the case of understanding limonene protection against colonic carcinogenesis, the studies were performed on rats and for shorter exposure time to the compound or its derivatives. Therefore, further in vivo models are required to determine the toxicity of the treatment for longer periods of time, as D-limonene is nontoxic but its alcohol derivatives could be toxic.

4.1. D-Limonene and Docetaxel (See [72]). Many combination studies are underway to determine an effective approach in treating advanced and aggressive prostate cancers. Docetaxel, a synthetic derivative of taxol, is primarily used to inhibit the microtubular structures in cancerous cells that support cell division. In addition to its role as a microtubule disruptive molecule, it has a host of inhibitory actions on genes which regulate cell proliferation, mitotic spindle formation, transcription factors, and oncogenesis. It also upregulates genes involved in apoptosis and cell cycle progression in prostate cancer. D-Limonene, discussed earlier has been shown to have anti-prostate carcinogenic effects at low dose concentrations. Logically; therefore, combining the two compounds may have a plethora of positive antitumor functionalities. In a study by Rabi and Bishayee [72], the combined treatment enhanced the sensitivity of DU145 prostate cancer cells that are known to be apoptotic resistant. This enhanced sensitivity was
thought to be mediated by reactive oxygen species (ROS) generation and activation of caspase 3 and 9. Such a positive in vitro outcome warrants further investigations in vivo, in models that mimic the progression of the disease, before it can be used in dietary supplements for therapy.

4.2. Lycopene and Fru/His (See [74, 111–114]). Serum lycopene (a carotenoid) levels have been shown to have an inverse correlation with prostate cancer risk. A diet-based population study showed that, of all the carotenoids assessed, high serum lycopene levels showed a statistically significant lower prostate cancer risk. Further analysis of their data revealed that lower serum lycopene levels in conjunction with β-carotene supplements were effective against lowering the risk of prostate cancer, suggestive for a combinatorial therapy [111]. Certain dietary compounds can be the source of cancer formation as seen with prostate cancer. It is believed that the nonfat portion of milk and excess calcium are some main factors in prostate cancer risk [112]. Numerous in vitro studies have shown that carotenoids have a greater influence in reducing tumors of the prostate origin, and lycopene and 1,2-dihydroxyvitamin D3 are at the forefront as risk reduction factors. In addition to their role as potent inhibitors of prostate cancer growth, they are biologically safe and cheaper forms of treatment. 1,2-dihydroxyvitamin D3 and lycopene have physiologically different roles, but combined they modulate pathways to synergistically inhibit proliferation and differentiation at much lower concentrations [113] and bear additive effects on cell cycle progression.

The assessments that lycopene is a safe dietary molecule with anticancer properties is supported by a number of population epidemiological and cohort-based
studies [112]. However, it is important to ensure that the statistical models used are able to adjust for many parameters for a true significant outcome. Regardless of the statistical model employed in these assessment studies, lycopene has emerged as a potent risk-reducing factor of prostate cancer and has been even supported by a study that was carried out across 28 countries. Intervention combination studies have not yet been performed. However, in vivo-based studies in mice models have shown that lycopene administered in the form of tomato powder and broccoli powder in a 10 : 10 ratio, increases its serum concentration to about 538 nM/g with about 0.4 nM/g concentrated in the prostate tissue itself. Diet-based intervention studies are required to determine the formulated diet required to improve the availability in the serum of patients and enhance the localized concentration of the molecule in the tissue. Such a diet-based treatment may serve as a suitable chemopreventive approach against prostate cancer or with patients at high-risk of the disease. Even though bioactive molecules successfully work in administering their protective functions in vitro, it appears through in vivo studies that diet and availability crucially dictate outcomes. A critical question to be asked is what factors constitute a perfect blend of bioactive mixtures. With the current research thus far, it is hard to address what the cut-off ratios are that need to be used in a diet that contain mixtures of potent nutraceuticals to coordinate similar effects clinically. Possibly a slight change in concentration of even one of the effective biomolecules may render the mixture ineffective in its function. It is rather an important task for pharmaceutical chemists and nutritionists to determine the ratios of effective biomolecules in a mixture and determine the pharmacokinetics and dynamics of that mixture.
Fru/His, a ketosamine, is also a derived product from tomatoes obtained by the reaction of a carbohydrate with an amino acid. This particular ketosamine has been found to assert chemopreventive effects by synergistically enhancing the activity of lycopene, by neutralizing ROS species and inhibiting DNA damage. Therefore, the complex of these two molecules may have a pivotal role in prostate cancer prevention. Although a rat model was used to determine the results of the treatment and pharmacokinetics of the compound are still not known, the combination of the two seemed to preferentially localize in the prostate more than in other tissues that were tested [74].

Occasionally, a combination may fail to incite anticarcinogenic effects as was seen by Mossine et al. [114]. Their experiments were conducted on the prostate adenocarcinoma rat model that was used by other groups, and their data had contradictory results to the effective action of lycopene itself and in conjunction with other micronutrients. Their study revealed that lycopene was not able to inhibit or reduce tumor load alone or in combination and that selenium alone in the mixture was able to induce antitumorigenic effects. Such outcomes are important as they open up more questions as to why a molecule that affects a given pathway behaves differently when tested within the same experimental model. Is it always dose or concentration or does molecule preparation and delivery impart effects on the efficacy of a drug?

4.3. Lycopene and Docetaxel (See [75]). Docetaxel is a potent chemotherapeutic drug that is clinically used to treat patients with advanced metastatic prostate cancers. Although the drug extends survival, it is for a very limited time period and with a poor prognosis. Lycopene, a natural compound, has been shown to have strong cancer
inhibitory properties against the prostate tissue. One study tested the possibility to use this combination of compounds to enhance survival of patients that were detected with aggressive, androgen-independent tumors. As predicted, docetaxel inhibited tumor growth in nude mice that bore tumor xenografts of human DU 145 cells. Analysis of molecular mechanisms revealed that the action of docetaxel was on regulating the insulin-like growth factor receptor (IGFR) pathway by suppressing IGF, and this effect was synergistically enhanced in the presence of lycopene. Together the molecules asserted negative downstream effects on Akt signaling pathways and suppressed survivin, products of which have been known to maintain tumor growth and enhance metastasis. Clinical trials using this combination may prove effective in treating patients that express high levels of IGFR in the prostate tumor and extend survival for a longer duration than what is possibly achieved by docetaxel alone, which is about 18–20 months.

5. **EGCG and Quercetin (See [31])**

EGCG exhibits strong chemopreventive and therapeutic activities as it influences many pathways as shown in Figure 1. Some of the mechanistic pathways are involved in regulating the levels of Bcl2, survivin, and XIAP and activation of caspase-3/7 to induce apoptosis. EGCG is also involved in inhibiting genes that are required for transition from epithelial to mesenchymal cells and retards migration and invasion which are primarily advantageous in terms of controlling aggressive tumors. EGCG mediates such synergistic actions in conjunction with quercetin to retard the self-renewal properties of cancer stem cells (CSCs), a population that, if inhibited, can influence tumor regression. Quercetin, a polyphenol, downregulates the expression of the heat shock protein (Hsp90) known to
influence apoptosis and growth inhibition of prostate tumors. Therefore, the combination of these molecules modulates their respective therapeutic effects to mediate synergistic growth retardation of CSCs. The study by Tang et al. [31] used relatively higher concentrations of EGCG (60 μM) in the presence of 20 μM quercetin. Probably concentrations of EGCG that can mediate similar synergistic levels, albeit at lower doses, need to be investigated, and the therapeutic potential across cancer stems cells of other origins need to be assessed if clinical applications are to be considered.

6. Resveratrol and Estrogen (See [39])

Selective estrogen receptor modulators that are used in the clinical treatment of breast cancers display dual agonist/antagonist effects in the tissues, especially in cancer initiation and progression. Drugs like tamoxifen emulate antagonistic effects on estrogen to contain the tumor. Agonistic-estrogen-like activity can in some instances enhance tumor progression which is not desired in most clinical treatments. Resveratrol, a polyphenolic compound abundant in grape skin and grape products including wine, is known to have chemopreventive properties as supported by numerous in vitro and in vivo studies. However, based on the experimental cell type, resveratrol induces either agonistic or antagonistic effects that can be weak or very pronounced. Resveratrol agonistic effects are totally reversed in the presence of estrogen, possibly mediated through estrogen receptor β. This reversal of effects is pertinent to prevention of breast cancer lesions in ducts that could become long-term neoplastic and cancerous. Of its many cancer protective functions, resveratrol in combination with glucan are potent immunomodulators by upregulating Cdc42 expression [115]. When natural compounds
exhibit multi-chemopreventive properties, conjugation therapies are advantageous over monotherapies. Albeit not clinically tested, harnessing cancer preventive and immune modulating functions of nutraceuticals seems to be a plausible approach to targeting hormonally independent aggressive tumors.

6.1. Resveratrol, Quercetin and Catechin (See Table 4 and [77]). The protective functions of polyphenols are manyfold. Numerous studies have analyzed their protective and therapeutic functions in vitro on tumor initiation that was chemically induced or in vivo via cellular implanted tumor formation. Few studies have established the functions of combined polyphenols on established tumors, as the majority of investigations have focused on individual mechanistic effects of the compounds. Dietary serum concentrations are influenced by the individual percentage of biomolecules present in the diet. Therefore, individual protective assessments of a compound show higher dose requirements, whilst mixtures may require lower doses to achieve the same effects. Additive and synergistic effects of compounds occur if their individual functions are enhanced in the presence of other molecules, perhaps by reinforcing the serum stability and availability of the various compounds in the mixture. Such observations were seen in both in vitro and in vivo testing of a mixture of three polyphenols, resveratrol, quercetin, and catechin, albeit pharmacokinetics studies are warranted.

6.2. Resveratrol and Cyclophosphamide (See [78]). Cyclophosphamide, a neoplastic drug, has a broad spectrum of activity on a variety of cancers, including breast cancers. The shortfall of the drug is its myriad of toxic effects on other systems. Dose reduction of
the compound would be a means of reducing its toxicity without compensating its anticarcinogenic activity. Resveratrol has been shown to successfully lower the effective dose of cyclophosphamide without altering its anticarcinogenic activity. Both of the compounds together synergistically enhance caspase-mediated cytotoxic activity, as demonstrated in MCF-7 cells, an aggressive breast cancer cell line (Table 4). The combination therapy resulted in the upregulation of p53, proapoptotic genes, Bax and Fas, and downregulation of antiapoptotic gene Bcl-2, suggestive of an apoptotic mechanism involved in cell death.

6.3. Resveratrol and n-Butyrate (See [79]). n-Butyrate is a short chain fatty acid produced by bacterial fermentation of fiber in the colon. The compound is a known differentiating agent and induces an epithelial phenotype in certain cultured cells. n-butyrate is a potent histone deacetylase (HDAC) inhibitor as well and one of its differentiation-inducing properties stems from its ability to inhibit HDACs. Resveratrol, discussed above, induces apoptosis through other mechanistic pathways. The combination of two bioactive molecules influencing apoptosis via different mechanistic pathways may associate to render an apoptotic phenotype in cancerous cells and inhibit tumor formation and progression. The 2 mM dose of n-butyrate used in the Wolter and Stein study [79] is probably much higher than what can be physiologically achieved. This dose is probably suitable for treatment of colon cancers where higher molar doses of n-butyrate are possible. However, n-butyrate is highly unstable, and its serum concentrations are lower than 2 mM. Since this molecule is a differentiating agent, its clinical use in treatment of other cancers is relevant. However, such therapies require
absolute lower effective doses and can probably be achieved by combining with molecules other than resveratrol or modifying the compound to specific conjugates to reach serum concentration levels.

6.4. Resveratrol and 5-Fluorouracil (5-FU) (See [80]). 5-fluorouracil inhibits thymidylate synthase, prevents DNA proliferation, and induces DNA damage-related apoptosis in colon cancer cells. Phase I clinical trials using a combination of resveratrol and grape powder have shown that resveratrol at low doses inhibit Wnt, a gene that is upregulated in colon cancers. Taking advantage of therapeutic effects of nutraceuticals, combined therapy of aforementioned resveratrol with 5-FU surfaces as a principal strategy in treating colon cancers. When used in combination, the presumption is that either additive or synergistic effects of the two could mediate tumor inhibition by modulating their individual apoptotic effects. The concern in using resveratrol is that higher concentrations of the doses are required in the treatment which clinically may not be reached through dietary consumption.

6.5. Genistein and Resveratrol (See [81]). Genistein and resveratrol as individual phytochemicals are very effective in the treatment and prevention of prostate cancer progression in rodent studies. Poorly differentiated prostate cancers often fail to respond to androgen-dependent treatments, and alternate treatments are required. Androgen receptors likewise have two functional roles, one as a tumor suppressor in normal prostate tissue and the other as an oncogene in neoplastic transformation, where it is altered either by mutations or DNA damage. Genistein and resveratrol used in an in vivo
rat-based study, modeled to understand the mechanistic action of combined treatments in the progression of prostate cancer, showed that they had more pronounced effects, albeit not synergistic. The statistically significant additive functions of reducing cell proliferation through mechanisms that regulate the androgen receptor levels and IGF-1, a biomarker found in patient serums with progressive and aggressive prostate cancers were achieved in combined therapies over the monotherapy regimes. Interestingly, the combination of genistein and resveratrol increased serum availability of both, but higher concentrations of resveratrol were achievable as compared to the single-dose regimen. Perhaps, absorption and stability of resveratrol were profoundly affected in a combined environment, which is clinically a clear advantage. The doses used in the study are physiologically safe and achievable in vivo by consumption of a soy-based diet high in the percentage of genistein. However, resveratrol is found in low levels in grape-based dietary products, and, therefore; a pure supplement of the compound is necessary in case that higher doses are required.

7. Genistein and Sulforaphane (SFN) (See [82]). Previous studies have shown that EGCG, a major polyphenol in green tea, can inhibit tumor growth through mechanisms that alter DNA methylation activity, reversing the expression of silenced genes involved in tumor inhibition in cancer cells. Hypomethylation of the promoters that are CpG-rich is more likely to be transcribed, with an exception of few like hTERT, the regulatory gene of telomerase [82, 116–119]. Epigenetics is a mechanism that has been studied for decades, and factors that regulate epigenetics are now believed to be very important as treatment possibilities in controlling tumors. DNA methylation and histone deacetylation
are well known epigenetic mechanisms that regulate many of the genes involved in cancers of various origins. Genistein combined with SFN, an histone deacetylase inhibitor (HDACI) has been successful in inducing the transcription of genes involved in regulating cell cycle by reversing the hypermethylated states of their promoters. This change was observed at low doses and was enhanced in the presence of sulforaphane more than that when genistein was used alone. However, in vivo studies of the same are warranted to determine epigenetic behavior of the dietary compounds before applications to human treatments are considered.

7.1. Benzylisothiocynate (BITC) and SFN (See [83]). BITC and sulforaphane are ITCs derived from cruciferous vegetables like broccoli. Individually both these molecules exert potent chemotherapeutic properties strongly supported by numerous studies. Oddly, even though both are isothiocyanates, they exert their therapeutic effects by controlling different pathways involved in tumorigenic inhibition. STAT3, a member of the STAT group of transcriptional factors, is required for early development and is dispensable in adult tissues. However, there appears to be a correlation between the constitutive expression of STAT3 and tumor development, indicative of its role as an oncogene. This gene appears to have important roles in cell proliferation, angiogenesis, and metastasis, a crucial requirement of tumor survival. Both BITC and sulforaphane have cancer inhibitory effects, affecting independent cell signaling pathways. However, the sequential combination of the two has been shown to regulate the STAT3 gene and others (Table 4), thereby, inducing apoptosis. How dietary molecules are presented to the cells in vitro is important to its cellular mechanistic actions. In the study by Hutzen et al. [83], sequential
addition of BITC to the cells after sulforaphane treatment was performed, which enhanced the reduction of STAT3 levels; however, simultaneous additions were not performed. Simultaneous additions would be important for any combination study to determine possible synergistic, additive, or antagonistic effects between the compounds. Preclinical studies should include various combinatorial interactions of the nutraceuticals being tested to determine the best way of using combined molecules in therapy.

7.2. SFN and Apigenin (See [84]). Phase I and Phase II enzymes are extremely important to cancer prevention. Dietary foods are sometimes modified to produce carcinogens through metabolism by the action of Phase I enzymes. Subsequently, the action of Phase II enzymes rapidly metabolizes these products to more soluble forms that are eliminated as body waste. Phase II enzymes are more concentrated in the duodenum and small intestine and less available in the colon. Increasing the availability of these enzymes in the colon can get rid of harmful carcinogens reducing the incidence of colon cancers, and, therefore, dietary supplements that induce Phase II enzymes would be promising tools for colon cancer prevention. SFN, an isothiocyanate, and apigenin, a flavanol, have independent cancer preventive functions. SFN is a strong inducer of UDP-27 glucuronyltransferase (UGT1A1). UGT1A1 is a major player in the detoxification process of carcinogens formed in the body and, therefore, is a potent Phase II enzyme. Treating nondifferentiated colon cells with a combination of SFN and apigenin was found to synergistically induce the expression of UGT1A1 suggesting a possible dietary tool for colon cancer prevention. The in vitro dose of the individual compounds used in the study was at physiological safe levels and can be easily achieved in vivo.
7.3. **SFN and 3,3′-Diindolylmethane (DIM) (See [85])**. The importance of investigating the roles of dose combinations on chemopreventive or therapeutic functions has been well dissected in a study by Pappa et al. [85]. Lower doses of SFN demonstrate antagonistic effects on cell proliferation and higher doses of both compounds had synergistic effects. Synergism of compounds is preferred if the outcome is tumor regression, but in clinical treatments synergistic actions should be mediated at safe lower concentrations rather than at cytotoxic levels. Presumably, the choice of compounds used, based on the genetic or cellular function required, is imperative to the success of the treatment. Possibly, SFN can synergistically inhibit the proliferation of cancer cells with compounds other than DIM at much lower doses, which has been investigated in studies using SFN with flavanols. This clearly highlights the problems of using combined therapies, especially since dosage is of critical importance for the success of clinical trials.

7.4. **SFN and Dibenzoylmethane (DMB) (See [28])**. When seeking for dietary molecules with potential chemoprotective and therapeutic properties, it is essential to understand how they mediate their combined action. Based on mechanistic studies, only compounds that are able to achieve synergistic or additive inhibitory or inductive actions on cellular genes, pathways, and/or phenotypes can then be chosen for treatment, even though their individual actions may be more pronounced. DMB is antimutagenic. Patients with aberrant polyp crypt (Apc) mutations are prone to spontaneously form aberrant polyps in their intestinal tissue, which later can transform to colorectal cancers. Treatment with DMB found in licorice can effectively inhibit such mutations in Apc, thereby protecting
individuals from aberrant polyp formations. This molecule, therefore, has potential in terms of colon cancer prevention.

SFN has a myriad of chemopreventive functions as seen before in other studies and in various tissues. A combination of these two chemopreventive agents will have a profound impact on individuals that are at high-risk or reduce the incidence of colon cancers through dietary supplementation. The study by Shen et al. [28] showed that dietary intake of SFN and DMB negatively influenced the incidence and number of tumors formed in the Apc mice. The combined doses used were half that of the individual doses. However, the observed effects were still synergistic at these doses. Interestingly, the serum and plasma levels of SFN and DBM were lower in the combined doses than when the compounds were used individually. Regardless of the low serum availability, the combination was able to mediate synergistic tumor inhibitory effects. This has important clinical significance as it is possible to achieve greater tumor toxic effects at low plasma concentrations. Mechanisms that influence such actions at low serum availability need to be further investigated.

8. Future Directions
Chemopreventive agents are much sought after as an early interventional approach to prevent tumor development or to lower the incidence risk of cancers. Given that the current available methods of treatment are chemotherapy, radiation, and surgery, all of which can induce significant side effects, an urgent need for alternate or adjuvant therapies has arisen. Phytochemicals are relatively safe and abundantly available from dietary sources. Therefore, alternate medicine aims at harnessing the protective properties
of these nonessential nutrients toward cancer prevention and treatment. A large database of studies supports the use of biomolecules in cancer treatment, albeit a majority of those are in vitro studies. Regardless of limited in vivo studies and clinical trials, phytochemicals show great promise in cancer treatment considering their safe use. Caution must be taken when addressing the efficacy of these molecules in clinical trials as many factors modulate their effects on cellular functions as detailed in Table 5. Combinatorial studies also show great promise, especially when lower nontoxic doses are required for prolonged periods to mediate potent chemotherapeutic functions with minimal side effects. Two of the major problems currently faced are dosage and delivery. To maintain a constant physiological serum dose availability, it is imperative that the agent is concentrated and stable in the tissue of concern. Combination technologies may be a solution to this problem. Nanotechnology is fast catching pace as the next level of technology in all spheres of science. Limited in vitro studies have shown that encapsulating dietary supplements in nanoparticles can effectively deliver the supplement and increase its stability and availability. Perhaps research needs to focus on such possibilities as avenues of using combination therapies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.
Acknowledgments

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GENERAL DISCUSSION

Diet-based combination therapies present a promising approach of treating cancers than monotherapies. Combination therapies are considered a more effective strategy by targeting the genes of interest of the same or different pathways [2]. The outcome, however, could be a synergistic or additive effect. In some instances, the combination may prove to be counter effective. Monotherapies against neoplasms have been successful in preclinical studies, yet in clinical trials either reduced to no effects are observed. The interaction of the molecule of interest with molecules in the host system may contribute to the reduced efficiency. Thus, the choice of the bioactive molecules and their combinatorial optimal dosage is crucial for an effective outcome. Combination epigenetic treatments are chosen such that each molecule in the combination has a specific epigenetic effect on the gene or genes of interest. Nonetheless, it can be difficult to separate epigenetic mechanisms as individual events since they now appear to function as cohesive mechanisms with cross-talk involved [32]. Combination treatment of cells can also provide insights into to how different dietary molecules interact in an in vivo environment.

We investigated the effects of the combination therapy of EGCG and NaB in colorectal cancers (CRCs). As epigenetic modulators, we postulate that EGCG and NaB combination therapy mediate demethylation and induce acetylation to re-express silenced genes in CRCs. Currently, surgery is the only form of treatment for advanced CRCs and therefore, inhibition of CRC cell growth should be considered as an added approach of
improving colon cancer treatment. Survivin, a member of the inhibitor of apoptosis (IAP), is highly expressed in most of the human tumors, including CRCs [33]. The differential expression of survivin in cancer versus normal tissues makes it an ideal diagnostic tool and a promising therapeutic target [34]. The mechanisms governing the upregulation of survivin are not fully understood, but wild-type (wt) p53 has been implicated in its regulation [35-39]. Disrupting the survivin induction pathway by bioactive molecules may become strategically important to promoting apoptosis and inhibiting tumor growth in CRCs. In our study, we evaluated the effectiveness of EGCG and NaB as a combination treatment in reducing survivin levels in CRCs, where the protein is highly expressed. Since survivin is considered to be a p53 responsive gene we investigated the effects of the combination treatment in p53-wt and p53-mutated cell lines. In our study, re-induction of nuclear p53 was observed in response to the combination treatment of EGCG and NaB. Induction of nuclear p21 was also observed, which in RKO CRCs was p53-dependent. However, since the combination treatment was equally effective in inhibiting HT-29 CRC cells, we believe that the combination is effective against CRC cells irrespective of p53 status.

Apart from its anti-apoptotic functions, survivin is also associated with the cell cycle in the G2/M phase [40]. The induction of p53 and p21 with the decrease in survivin resulted in cell cycle arrest in the G2/M phase for RKO and HCT-116 CRC cells and G1 phase in HT-29 CRC cells. Therefore, the anti-cancer effects of EGCG in RKO, HCT-116 and HT-29 are most likely to occur through a pathway that inhibits survivin and induces p21 in conjunction with NaB. The significant decrease in survivin as observed in our study of all three colorectal cancer cell lines is encouraging of the chemotherapeutic
effects of the combination treatment. This is an important finding as the combination treatment of EGCG and NaB induces a dual effect targeting apoptosis and cell cycle arrest through the reduction of survivin expression.

We believe that EGCG and NaB are effective in inducing genes that regulate cell division and apoptosis through changes of the epigenome of CRCs through their inherent epigenetic modulation abilities. Examination of DNMT1 and HDAC1 levels after treatment showed significant reduction in the combination treatment as compared to the individual treatment. Although EGCG and NaB are potent DNMT1 and HDAC1 inhibitors, respectively, our data suggest that there is a possibility of crosstalk involved in inducing additive effects. Studies have also shown that HDAC1 is highly expressed in a subset of colorectal carcinomas and that high levels of HDAC indicate reduced patient survival in colon cancers [26]. Inhibition of HDAC1 has been shown to promote reduced cell growth in colon cancer cells and such an observation was supported by our colonogenic assay studies in RKO CRC cells [41]. Therefore, inhibiting HDAC1 and survivin together by the combination treatment of epigenetic inhibitors can greatly improve the prognosis and survival of patients with CRC.

From our investigation, the combination treatment of EGCG and NaB appears to regulate more than one signaling pathway to induce CRC apoptosis and inhibit cell growth. In addition to regulating the survivin/p53 pathway, the increase in the mRNA and nuclear protein levels of NF-κB-p65 as observed in our study is suggestive of the involvement of the NF-κB pathway in mediating chemotherapeutic effects of the combination treatment. NF-κB-p65 is also activated in the presence of DNA damage and requires HDAC1 for the DNA repair process [42]. Our results showed that EGCG and
NaB as a combination reduced the levels of HDAC1 and increased NF-κB-p65 nuclear levels in RKO CRC cells. We postulate that the reduced levels of HDAC1 by the combination treatment followed by DNA damage as evident by the increase in gamma-H2AX levels further enhanced the inhibition of RKO CRC growth via the NF-κB-p65 pathway. Taken together, increase in DNA damage, induction of cell cycle arrest and apoptosis, inhibits CRC cell growth and is mediated by the survivin/p53 pathway and NF-κB-p65 pathway.

EGCG and NaB as a combination have tremendous potential in the treatment of CRCs. Though EGCG has important anti-cancer properties, the major limitation of this polyphenol is its bioavailability and stability [2, 43]. EGCG undergoes rapid biotransformation in the digestive system by methylation, glucuronidation, and sulfation [2]. However, despite low plasma concentrations, a large amount of conjugated and free EGCG makes it way to the colon [44]. In the colon, EGCG conjugates are deconjugated by microbial activity increasing the availability in the colon. EGCG has also been shown to have prebiotic-like functions modulating the colonic microbial landscape and enhancing the production of short chain fatty acids (SCFAs) [44, 45]. Of the two bioactive molecules, NaB is more readily available to the colon through dietary consumption or fermentation of dietary fiber by microbes in the colon. Nevertheless, as a combination, strategies for further improving EGCG and NaB availability and stability at concentrations that promote chemotherapeutic effects need to be considered. Previous studies have shown that the prodrug form of EGCG not only enhances its stability but improves its health promoting benefits [46-48]. Similarly, butyrate delivered in the form of tributyrin shows remarkable and improved chemotherapeutic effects [49-57].
Therefore, designing the delivery of EGCG and NaB as a complex is important for CRC treatments. In the future, preparation of EGCG and NaB as formulated edible nanoparticles may allow for oral consumption of the bioactive molecules with improved stabilities and immediate availability [58, 59].

The work presented in this dissertation was able to show that combination therapies have great potential if the optimal combination and dose are selected. EGCG and NaB as a combination therapy can significantly inhibit colon cancer cell growth by targeting and inhibiting specific genes that promote cell division and repair and involve epigenetic mechanisms. The findings from this study are important as they provide insights into the mechanistic action of the combination therapies in colon cancer treatment. Nonetheless, studies that assess DNA protein interactions would provide additional insights into the pathways affected by the combination treatment leading to the observed changes in gene expression. Many pathways could be involved in the tumorigenesis of colon cancer and therefore single gene-protein changes by the combination treatment although informative and helpful, may not fully illuminate the multiple pathways affected by the treatment. These could be possible future studies where entire genomes are evaluated for the DNA-protein interactions induced by combination treatments through chromatin immunoprecipitation sequencing (ChIP-seq) analysis.
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APPENDIX A

ENVIRONMENTAL EFFECTS ON AGE-ASSOCIATED EPIGENETICS

by

SABITA N SALDANHA, ASHLEY MCCOLLUM AND TRYGVE TOLLEFSBOL

Epigenetics of Aging

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Format adapted for dissertation
**Abstract** Aging of an organism appears to be a precursor for most, if not all disease outcomes. However, some diseases associated with aging are due to genetic mutations, while others occur even without altering the genetic sequence, through a phenomenon known as epigenetics. DNA methylation and histone modifications are classic examples of epigenetic modifications. These modifications appear to be in a constant flux and, therefore, can be altered by exposure to environmental insults over the life time of the organism. This chapter focuses on how environmental conditions such as heat stress, exposure to metals, and nurturing can affect the dynamic epigenetic modifications in aging. In addition, how these modifications can either directly or indirectly result in age-associated pathologies are also addressed.

**Keywords:** Epigenetics · Aging · Environment and Epigenetic modifications.

**Introduction**
Organismal systems are comprised of three types of cell populations. The stem cell populations that primarily give rise to new cells, the differentiated cell populations that are tissue and function specific, and apoptotic populations composed of dead cells. Differentiated cell populations are of particular interest as these populations are responsible for maintaining the functional integrity of the organs. Differentiated somatic cells, however, have a finite life span and follow Hayflick’s limit (Hayflick 1979, 1980, 1985), that is, after a programmed number of cell divisions, the cells are unable to divide and thereby enter the senescent phase. Senescent cell populations are the hallmark of the aging process and may induce the aging phenotype and in some cases age-associated
pathologies (Price et al. 2002; Wiemann et al. 2002; Minamino and Komuro 2007) (Fig. 1). Several other determinants contribute to the aging process which are discussed in other reviews (Sedivy et al. 2008). It is interesting to note that although several molecular changes within a cell contribute to the aging phenomenon, of particular interest is the change in gene expression as these fundamental units control almost all cellular processes. The previously held belief that mutations solely affect gene expression has been refuted. Recent investigations have shown otherwise (Sedivy et al. 2008). Altered gene expression can be mediated by an epigenetic process where the sequence of the gene remains unaltered, and the patterns of these changes are stably inherited through cellular divisions. Stable inheritance of these patterns is controlled by mechanisms such as DNA methylation and post-translational modifications of key histone residues (Fraga and Esteller 2007). The resultant accumulation of certain epigenetic marks and the interplay of these events may influence how an organism ages (Fraga and Esteller 2007). Breaking this code can help to understand what marks trigger a cell to age or proliferate. The most predominant factors known to influence such epigenetic events are the environment and nutrition (Fraga et al. 2005; Feil 2006; Fraga and Esteller 2007).

DNA Methylation

DNA methylation is a predominant epigenetic mechanism that controls gene expression. Methylation occurs at CpG dinucleotide residues controlled by the DNA methyltransferase enzymes (Feil 2006). The methylation patterns that are inherited are substrate specific, and maintenance methylation by DNA methyltransferase 1 (DNMT1) will occur only if a premethylated template exists. This allows for a faithful reproduction
Fig. 1 Cellular and tissue changes occurring with the onset of aging. The circles (light gray) symbolize mitotically active and proliferating cells as seen in young tissues. However, an internal genetic clock causes the cells to switch from mitotically active to slow proliferating cells as seen in adult tissue, with predominant silent but metabolically active senescent cells (middle gray, spindle shaped). This phase deteriorates further, and genes responsible for the healthy sustenance of cells may be silenced (e.g., housekeeping genes). Eventually cell death (dark gray, star shaped) ensues which may trigger many age-related diseases.
of the previous methylation pattern, without altering the epigenetic mark. Methylation of CpG dinucleotides is primarily associated with gene repression. Observations of global hypomethylation have been documented in some studies (Wilson and Jones 1983; Mays-Hoopes 1989; Richardson 2003; Liu et al. 2007). However, hypermethylation of locus-specific genes or specific promoter elements can induce gene expression (Feil 2006). Epigenesis is not a simple process but has some degree of complexity, as seen in higher order organisms. The epigenetic process of DNA methylation in gene control has been well established in X-chromosome inactivation and genomic imprinting (Feil 2006).

X-Chromosome Inactivation

Although very few phenotypic genes are present on the X chromosome, the genes on this chromosome control many developmental processes, and thus, maintaining the dosage is of utmost importance. X-chromosome inactivation occurs at the X inactivating center (XIC) (Avner and Heard 2001; Boumil and Lee 2001; Heard et al. 2001; Sado et al. 2001). Inactivation of X (Xi) chromosome is mediated through epigenetic intervention (Wang et al. 2001). The inactivation of X takes place randomly in the somatic cells, and only in the germ line of the females are both X chromosomes active. Inactivation of the X chromosome is initiated at the X-chromosome inactivating center and is mediated by a sole gene Xist (X inactive signal transcript) (Avner and Heard 2001; Salstrom 2007; Vincent-Salomon et al. 2007). An RNA molecule transcribed by this gene covers the X chromosomes and initiates the wave of inactivation across the whole chromosome with the exception of a few regions. In addition to the specific RNA transcript that is not translated, heterochromatization of the X chromosome is mediated by DNA methylation
and chromatin remodeling. Modification of histone H3 tails accounts for some of the early events that take place once the X chromosome that is marked for inactivation by the Xist RNA (Heard et al. 2001). Lysine 9 and 4 of histone H3 becomes globally methylated and hypoacetylated, and hypomethylated, respectively (Heard et al. 2001). Methylation of lysine 9 may take part in the chromatin reorganization induced by Xist transcript that stimulates the repression of X chromosome. HP1 heterochromatin proteins have the ability to bind to H3 methylated K9 and to bind to histone methyltransferases that enhance the spread of methylation (Nakayama et al. 2001). This allows for the spread of the inactivation state. Histone H4 hypoacetylation on the other hand is a modification that occurs as a later event (Kaneshiro et al. 2007).

Table 1. Histone modifications involved in X chromosome and gene activation/inactivation.

<table>
<thead>
<tr>
<th>Histone modified</th>
<th>Residue modified</th>
<th>Type of modification</th>
<th>Effect on X chromosome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>Lysine 27</td>
<td>Trimethylation</td>
<td>Silences certain regions on X chromosome and may associate with some coding regions</td>
<td>Brinkman et al. (2006)</td>
</tr>
<tr>
<td>H3</td>
<td>-</td>
<td>Acetylation</td>
<td>Associated with promoters of active genes on X chromosome (but may not be transcribed)</td>
<td>Brinkman et al. (2006)</td>
</tr>
<tr>
<td>H4</td>
<td>-</td>
<td>Acetylation</td>
<td>Associated with promoters of active genes on X chromosome (but may not be transcribed)</td>
<td>Brinkman et al. (2006)</td>
</tr>
<tr>
<td>H3</td>
<td>Lysine 4</td>
<td>Trimethylation</td>
<td>Associated with promoters of active genes on X chromosome</td>
<td>Brinkman et al. (2006)</td>
</tr>
</tbody>
</table>
This later event suggests that early H3 histone modifications are primarily responsible for chromosome-wide inactivation (Heard et al. 2001; Jenuwein and Allis 2001). Studies have shown that the Xi chromosome has interspersed regions of active and inactive regions. This indicates that the inactivation is not signaled by individual histone marks but rather the cumulative effects of various histone modifications acting together (Table 1). What has been observed is that there are several genes on the inactive X that escape inactivation (Kaneshiro et al. 2007). A few that are silenced are housekeeping genes such as Hypoxanthine phosphoribosyltransferase 1 (HPRT1) and phosphoglycerate kinase 1 (PGK1). The genes are silenced through epigenetic mechanisms of DNA methylation as the promoters of these genes are CpG rich. Many of these genes also control various cellular processes. The inactivation and silencing of genes occur early in embryonic development (Kaneshiro et al. 2007). However, reprogramming of these genes can be achieved through environmental influences as the epigenome is quite dynamic. The exact relationship between environmental factors and X reactivation via epigenetic mechanisms yet needs to be ascertained. The speculation is that with age, probably this stability can be
reprogrammed or erased resulting in reactivation that could possibly trigger or amplify phenotypic defects.

**Histone Modifications**

Histones are basic proteins that are evolutionary well conserved and are essential to the proper packaging of DNA in the nucleus. About ~146 bp of DNA are wrapped around the histone octamer molecule which is comprised of two subunits each of H2A, H2B, H3, and H4 (Luger et al. 1997; Jenuwein and Allis 2001). This structure is called the nucleosome and forms the repeating unit of chromatin. The activation and inactivation of gene function has been established by the reversible chemical modification of histone amino ‘tails’ that initiate euchromatic or heterochromatic states. Commonly, acetylation, phosphorylation, methylation, ubiquitination, and sumoylation are involved in modifying the exposed histone sequences (Jenuwein and Allis 2001; Ke et al. 2006; Iniguez-Lluhi 2006; Sims et al. 2006). Amino acid residues such as lysine and arginine are prone to such modifications. However, the degree of chemical modification, the number of residues modified, and the type of modification greatly influence the control on gene expression. For example, two processes such as acetylation and methylation of histone residues can act simultaneously either on identical or different subunits, but the overall pattern or code dictates if a gene is turned on or off. The modified histone states are initiated by enzymes such as histone methyltransferases (add methyl groups), histone acetylases [HATs] (add acetyl groups), or histone deacetylases [HDACs] (remove acetyl groups). Investigations have demonstrated that a cross talk can exist between epigenetics states (Vaissiere et al. 2008). Histone acetylation allows the unwinding of DNA
associated with the nucleosome exposing it to regulatory elements. Thus, acetylation not only alters the structure of chromatin but also can dictate whether a gene is expressed or repressed based on the regulatory factors that can bind to exposed promoter elements. Although histone acetylation and its role in carcinogenesis is intensively studied, relatively little is known about the molecular mechanisms by which this epigenetic process influences changes in the histone code in aging. One can speculate that in young healthy cells, loss of methylation of CpG islands with acetylation maintains the expression of crucial genes. However, with age and exposure to environmental insults, a shift in epigenetic profile may occur whereby the spread of methylation to CpG residues with deacetylation of local histones can induce progressive loss of gene expression (Vaissiere et al. 2008). This shift in profiles may enhance age-related etiologies (Cairns 2001; Rowley 1998; Wolfe 2001; Vaissiere et al. 2008).

Environmental Effects and Epigenetics

Organisms are constantly exposed to environmental insults throughout their lifetime that can inadvertently change the landscape of molecular processes. Environmentally induced stresses contributed by an individual’s occupation, lifestyle, and external surroundings are considered to be extremely crucial to these changes. Exposure to metals such as nickel, lead, cadmium, arsenic are known to initiate tumorigenesis (Herceg 2007; Vaissiere et al. 2008). These factors therefore have a potential effect on chromatin organization, mediated by DNA methylation and histone alterations that code specific epigenetic information. These metals therefore can significantly affect changes in normal epigenetic patterns, initiate aberrant epigenetic signaling, and induce neoplasms. There is
a well-established link between ionizing radiations and chromosomal instability. This instability most probably arises from abnormal methylation of unmethylated regions. Chronic exposure to UV radiation has been shown to induce global hypomethylation as seen in studies carried out in mice (Herceg 2007). The end result of these abnormalities early on in life culminates in age-associated malignancies.

**Nickel**

Epigenetic damage by nickel particles occurs in heterochromatic regions of chromosomes. Studies have shown that nickel tends to increase DNA methylation near heterochromatin (Herceg 2007). Interestingly, tumor suppressor genes and senescence genes may be located in these regions. Therefore, critical cellular genes can be turned off by de novo methylation of the promoters. Sometimes more than a single epigenetic event can transcriptionally silence important genes. This has been seen in a gene such as guanine-hypoxanthine phosphoribosyltransferase (gpt). This gene is transcriptionally silenced not only by DNA methylation but also by a decrease in global acetylation of histones H3 and H4 and increased histone H3K9 dimethylation (Herceg 2007). Studies have shown in the presence of nickel that specific lysine residues are hypoacetylated, primarily at position 12 and 16 of histone H4 (Broday et al. 1999, 2000; Zoroddu et al. 2000. In addition, nickel preferentially binds to a site at histidine 18 (Broday et al. 2000). This binding of nickel to histidine at position 18 prevents or hinders the accessibility of neighboring lysine residues to the HAT complex. Thus histone code patterns are significantly modified or changed by chronic exposure to the nickel molecule (Table 2).
Table 2. Changes in histone modification by exposure to nickel

<table>
<thead>
<tr>
<th>Histone Subunit</th>
<th>Epigenetic Modification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2A</td>
<td>Cleaves C-terminal tail; increases ubiquitination</td>
<td>Bal et al. (2000a, b); Karaczyn et al. (2003); Ke et al. (2006)</td>
</tr>
<tr>
<td>H2B</td>
<td>Truncates, deaminates and oxidizes; increases ubiquitination</td>
<td>Kaneshiro et al. (2007)</td>
</tr>
<tr>
<td>H3</td>
<td>Increase in lysine 9 dimethylation, decrease in acetylation</td>
<td>Chen et al. (2006)</td>
</tr>
<tr>
<td>H4</td>
<td>Decrease in acetylation of lysines 12 and 16 respectively.</td>
<td>Broday et al. (2000)</td>
</tr>
</tbody>
</table>

Arsenic and Cadmium

Drinking water and food contain low but permissible levels of arsenic. However, chronic exposure of this element can exert perturbations in epigenetic patterns. Arsenic-related hypomethylation is due to the depletion of the cofactor S-adenosylmethionine, required for methylation as well as arsenic metabolism. The sodium form of arsenic has been shown to increase genomic hypomethylation (Zhao et al. 1997) of certain crucial genes such as Harvey rat sarcoma viral oncogene (Ha-Ras), and these perturbations can allow for neoplastic development later on in an aged individual. Cadmium inhibits the epigenetic modifying DNA methyltransferases leading to hypomethylation. However, prolonged exposure to cadmium stimulates regional hypermethylation and global hypomethylation which is a commonly observed phenomenon in aged tissues.
**Lead (Pb)**

The brain undergoes numerous changes throughout life in humans. The set of genes that are passed along from each generation has much to do with the direction of aging in the brain, but environmental influences can contribute to the process as well. For example, the structure of chromatin can be influenced by the environment which can cause a lifetime of phenotypical changes and possibly be passed on to future generations. Diseases such as Alzheimer’s and Parkinsons are prime examples of environmental effects on phenotype (Miller and O’Callaghan 2008). Since some of the diseases have a later onset of the phenotype, one can speculate that the genes are initially silenced until affected by an environmental factor, which may reverse the inactivation through epigenetic mechanisms. Methylation is one such epigenetic element that partakes in altering the epigenome. DNA methylation can be affected by environmental factors, such as drugs, maternal care, and social situations, thus confirming the association of DNA methylation to the long-term changes of gene expressions in the brain (Champagne and Meaney 2007; Champagne 2008; Champagne and Curley 2008). Exposure to lead early in development is known to have effects on brain development (Zawia and Basha 2005, Wu et al. 2008). Brain development normally occurs during early pre- and postnatal periods followed by further development until early adulthood. Lead-mediated changes in brain development have been associated with specific genes such as amyloid precursor proteins (Wu et al. 2008). In normal aging individuals, the accumulation of amyloid plaques and associated proteins has been observed, and this process if accelerated can induce neurodegenerative diseases such as Alzheimer’s disease. Exposure to lead decreases methylation of a crucial few genes involved in brain development such as the
amyloid beta (A4) precursor protein (APP) gene (Wu et al. 2008). Hypomethylation of the APP promoter leads to decrease in the methylation burden, thereby inducing APP mRNA production. Consequently the increase in APP production is associated with an increase in the amyloidgenic β cleavage product during senescence. This cleavage product forms aggregates and generates free radicals that attack biomolecules such as DNA. The presence of a methylated cytosine impairs the repair of adjacent oxidized guanine bases, thereby increasing the susceptibility of neurons to further damage. Events such as these may collectively enhance the neurodegenerative process. Maternal behavior appears to play a significant role in altering behavior and physiology. One study (Meaney and Szyf 2005) demonstrated that maternal nurture influenced epigenomic patterns, especially of the brain. This group analyzed the epigenetic patterns of a specific gene, the glucocorticoid receptor. Differential methylation patterns of the promoter of the glucocorticoid receptor were observed in the hippocampus region, dependent on the type of nurture provided (grooming, licking, arched back feeding) (Meaney and Szyf 2005). In addition, the expression of nerve growth factor-induced clone A (NGFIA) that binds to the promoter region of the glucocorticoid receptor is altered by histone acetylation (Weaver et al. 2004a, b). Nevertheless, these marks and consequently the effect of the maternal influence can be reversed through pharmacological interventions indicating that the marks are dynamic rather than stable.

**Heat Stress**

A few studies have shown that heat stress may induce changes in methylation imprinting patterns (Zhu et al. 2008). However, these changes may be specific to only a few genes
involved in embryo implantation and development. Aberrant imprinting patterns have been shown to cause drastic developmental failures in embryos exposed to heat stress (Ealy et al. 1995, Ozawa et al. 2002). Imprinting of genes is primarily controlled by a well-known epigenetic mechanism, DNA methylation. Genomic demethylation and remethylation occur in germ cell development and after fertilization (Reik et al. 1990, Howlett and Reik 1991). However, the methylation patterns acquired are maintained after fertilization and preimplantation development. Thus, these patterns which are otherwise maintained in normal methylation imprinting may be lost due to heat stress. To study the effect of heat stress and its effects on methylation patterns, some groups have assessed four imprinted genes, two paternal and two maternal genes (Lau et al. 1994; Lefebvre et al. 1998; Zhu et al. 2008). These studies showed that the paternal imprinted genes H19 and imprinted maternally expressed transcript (non protein coding) (H19) insulin-like growth factor 2 receptor (Igf2r) and the maternal imprinted genes parternally expressed gene 1 (Peg1) and paternally expressed gene 2 (Peg2) reveal a possible link between heat stress and methylation imprinting (Lau et al. 1994; Lefebvre et al. 1998; Zhu et al. 2008). Surprisingly, heat stress-related abnormal methylation imprinting has been shown to affect paternal imprinting rather than maternal (Zhu et al. 2008). But this evidence is limited to only a few genes. For a more concrete conclusion several imprinted genes need to be analyzed. One can speculate that under heat stress, changes may occur in the maternal body. These changes may induce intracellular oxidative damage that can affect sensitive biomolecules such as DNA (Zhu et al. 2008). However, a clear-cut relationship between heat stress and epigenetic changes in the embryo is still unclear. The paternally imprinted genes H19 and Igf2r tend to be hypomethylated under heat stress, which has
been supported by several studies (Doherty et al. 2000; Khosla et al. 2001a, b; Zhu et al. 2008). Abnormal methylation imprinting may affect phenotypic outcomes of the genes resulting in developmental errors. Maternally imprinted genes, at least those that have been studied, have been shown to be unaffected by heat stress (Zhu et al. 2008). However, a large body of evidence is required to support these observations. Therefore, extensive investigations need to be carried out to discern the exact changes in epigenetic patterns induced by heat stress. These patterns may differ from what are seen in vitro versus in vivo.

*Environment and Epigenetic Drift in Populations*

In the approach of understanding how environment affects the epigenetic mechanisms of populations as a whole, identical genomes are analyzed for any subtle or drastic changes based on geographical location, lifestyle, or environment. This assessment has been investigated in monozygotic twin population studies (Petronis 2006). Thus any changes in the phenotype of a given trait in identical genomes can be attributed as an environmental contribution. Although biases may exist, in large-scale epigenetic studies of twin populations, these studies can provide a means of understanding the impact of environment on gene expression, the genome, and organism as a whole (Petronis 2006). Studies and observations in different organisms have shown the effect of environment on chromatin modifications. For example, plants exposed to cold temperatures fail to flower and this phenomenon is mediated by histone modifications to genes that encode repressors of flowering (Amasino 2004; Sung and Amasino 2006). Environmental stress can bring about expression of previously silenced genes, which has been observed in
Drosophila studies. During stress, a specific heat shock chaperon protein Hsp90 increases the activity of histone H3 lysine 4 methyltransferase, inducing the expression of specific target genes (Ruden et al. 2005). Extensive grooming which includes licking and arched back nursing have shown to alter the epigenetic patterns in the promoter of the glucocorticoid receptor in the hippocampus of the pups (Weaver et al. 2004a, b). All these studies indicate that epigenetic modifications serve as a substrate for the environmental insults. Thus, through these studies, assessment of molecular epigenetic changes mediated by environmental factors can be assessed. Comparison of identical twins for testing environmental epigenetics is highly suitable as their DNA sequences are identical and any change or discordance seen in a given phenotype may be attributed to the environmental factor. Biases and multi-environmental factors can change the overall scenario; however, initial studies using these cohorts can provide a wealth of information. For example, a study which analyzed the Beckwith–Wiedemann syndrome demonstrated that imperfect imprinting of a gene at KCNQ1OT1 (KCNQ1 overlapping transcript 1) due to DNA methylation differences in monozygotic twins (MZ) affected by the syndrome was the cause for this disorder (Weksberg et al. 2002). Therefore, lack of maintenance methylation at a key stage of embryogenesis is a factor for the observed phenomenon. Similar studies that used MZ twins to assess global and locus-specific epigenetic differences showed that in a cohort ranging from 3 to 74 years of age, an age-specific epigenetic drift was observed (Fraga et al. 2005). However, the discordance in the onset of disease does not always correlate with the age of onset (e.g., Alzheimer’s versus diabetes mellitus type 1). Twin studies revealed that the concordance of Alzheimer’s disease was 83% but that of diabetes type 1 was as low as 23% (Petronis
Further studies are required to analyze the implication of DNA sequence differences as compared to epigenetic differences in the contribution to the synchronous/asynchronous nature of the onset of the disease.

**Conclusion**

Nurture and nature tend to influence the physiological and behavioral outcomes of individuals. The observation may be attributed to the effects of environment, both extrinsic and intrinsic, on differential developmental patterns. Epigenomic marks are relatively stable and occur early in the developmental program. These stably inherited patterns, however, can be changed or reversed by environmental influences such as stress, toxic metal exposure, or even maternal nurture. Understanding these epigenetic marks is crucial to knowing the patterns associated with aging cells or if particular codes are associated with diseased phenotypes. Since aging epigenetics is a newly explored field, well-structured studies are required to understand the interplay of individual or coordinated codes on gene expression mediated by environmental cues.

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APPENDIX B

PATHWAY MODULATIONS AND EPIGENETIC ALTERATIONS IN OVARIAN TUMOR BIOGENESIS

by

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Abstract

Cellular pathways are numerous and are highly integrated in function in the control of cellular systems. They collectively regulate cell division, proliferation, survival and apoptosis of cells and mutagenesis of key genes that control these pathways can initiate neoplastic transformations. Understanding these pathways is crucial to future therapeutic and preventive strategies of the disease. Ovarian cancers are of three major types; epithelial, germ-cell, and stromal. However, ovarian cancers of epithelial origin, arising from the mesothelium, are the predominant form. Of the subtypes of ovarian cancer, the high-grade serous tumors are fatal, with low survival rate due to late detection and poor response to treatments. Close examination of preserved ovarian tissues and in vitro studies have provided insights into the mechanistic changes occurring in cells mediated by a few key genes. This review will focus on pathways and key genes of the pathways that are mutated or have aberrant functions in the pathology of ovarian cancer. Non-genetic mechanisms that are gaining prominence in the pathology of ovarian cancer, miRNAs and epigenetics, will also be discussed in the review.

Abbreviations: CDK, cyclin dependent kinases; p27kip1, cyclindependent kinase inhibitor1B; p16INK4a, inhibitor of kinases 4a; KRAS, Kirsten rat sarcoma oncogene; NF-kB, nuclear factor kappa B; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog; E2F, transcription factor.

Introduction

Ovarian neoplasms are challenging to detect and their genesis is often considered as de novo or sporadic (Scully, 1995; Liu and Ganesan, 2002; Zikan et al., 2007; Weberpals et
al., 2008, 2011; Sarojini et al., 2012; Wysham et al., 2012). When detected the cancer is often advanced and is at stage III/IV category (Kurman and Shih Ie, 2010). The absence of or poor detection methods of precancerous lesions of the tissue account for the late detection of ovarian cancer (Fleischer et al., 2012; Sarojini et al., 2012). Thus, ovarian cancer is considered to be a very lethal disease of gynecological origin.

In contrast to previous reports, ovarian cancer is not considered a single disease of epithelial origin, but instead covers a group of tumors that are morphologically and genetically distinct (Kurman and Shih Ie, 2010, 2011). The hypothesis however is still highly debated. Some of the reasons include late detection, inability to detect precancerous lesions and therefore insufficient evidence of neoplastic origination. This obscurity in the pathogenesis of the disease requires the understanding of the molecular aspects that influence the cellular pathways of the ovarian tissue. Based on morphology, genetics, and site of origination, ovarian cancers of epithelial-cell origin have been categorized into two groups. The type I group are those that are strictly confined to the ovary and are low-grade serous, endometrial, mucinous, and clear-cell type (Fig. 1; Kurman and Shih Ie, 2010, 2011; Le Page, 2010). These tumors are genetically more stable, have few to rare p53 mutations, are easily diagnosed and have a good prognosis. However, only 25% of the ovarian cancers detected are of this type. The Type 2 group contains tumors that are aggressive and comprise highgrade serous carcinomas, undifferentiated carcinomas and carcinosarcomas (Fig. 1; Le Page, 2010). These tumors
Fig. 1. Proposed theories of origination of epithelial-cell ovarian cancers (EOCs). The primary ovarian cancers reside in the ovary tissue itself. However, cells from the endometrium, fallopian tube, stroma can give rise to the histological types of tumors of epithelial-cell origin. As shown in the diagrams, 1a, 1b, and 1c denote the commonly seen ovarian tumors epithelia, stroma, and germ-cell tumors, respectively. However, EOCs are the predominant form and closer examination reveals that the origin could stem from other reproductive tissues such as the endometrium lining the uterus, cervical tissue or from the epithelia of the fallopian tube. Retrograde menstruation problems can be a pathway for the transfer of endometrial cells to that of the ovary. Ovulation and encystment of cells can be a mode of travel for cells from the fallopian tube. Therefore, the serous type EOCs arise from the fallopian tube (2a); the endometroid EOCs arise from the cervical or uterine tissue (2a and 2b); the mucinous and clear-cell tumors could arise from cells of 1b, 1c, (internal environment of the ovary) and 2a (cervical tissue). Hence, ovarian epithelial tumors are of the multi-histological type. Symbols used: grey filled cloud-like structure, tumors; small open oval circles, follicles in the ovary.
constitute 75% of the ovarian cancers with a 90% death rate and the site of origination stems from tissues other than the ovary. These tumors exhibit genetic instabilities with a higher percentage of p53 mutations (Kurman and Shih Ie, 2011).

Since the last 30 years or so, many preclinical and clinical trials have been conducted to determine the therapeutic efficacies of drugs against ovarian tumors. However, the success rates against ovarian cancers have been few. A majority of tumors that are removed through cytoreductive surgery resurface and become aggressive and have been proposed to be mediated in part through the treatment itself (Steffensen et al., 2011a; Modugno and Edwards, 2012; Stathopoulos et al., 2012). Elucidating the underlying factors that govern the genesis, progression and metastasis of this disease would provide valuable information for the development of diagnostic, prognostic and therapeutic approaches of the disease. Further understanding the genes and pathways that become dysregulated either due to loss of heterozygosity, loss of function, amplification or mutation would facilitate progress in managing ovarian cancer. This review will focus on the pathways deregulated and instrumental in the pathogenesis of ovarian tumors and key genes that are involved in these pathways. Genetic and epigenetic factors that contribute to these changes will also be analyzed.

**Genes Involved in the Biogenesis of Ovarian Cancers**

**p53**

Mutations in cell-cycle regulatory genes, primarily tumor suppressor genes, favor an uninhibited growth of cells and serve as precursors of immortality. Combined mutations in these genes and oncogenes encourage a neoplastic phenotype. How these tight
regulations become deregulated through mutations and how these mutations affect important regulatory pathways is important to a diagnostic and prognostic approach for all cancers. Unlike cancers of other origins, a majority of ovarian cancers are of epithelial-cell origin, commonly designated as epithelial ovarian carcinomas (EOCs). Nonetheless this single layer epithelia morphs to give rise to histologically different types (Kurman and Shih Ie, 2011). The transition is thought to occur through the cell cycle and genes that control metabolic and molecular pathways within the ovarian cell or via changes of the epithelia of surrounding reproductive tissue that find their way into the ovary through the inflammation and repair process of ovulation (Figs. 1 and 2).

**Fig. 2.** Cell cycle pathways involved in ovarian carcinogenesis. The cell cycle traverses through the G1-S-G2-M phases tightly controlled by check points between each phase through a set of regulatory proteins, primarily cyclins and kinases. The levels and expression of these regulators ensure the fate of the preceding and proceeding phases of the cycle. Cellular destinations, division, differentiation, senescence and apoptosis are
controlled by a certain restriction in the late G1 phase termed the restriction (R) point. It is when cells successfully cross this threshold point, that cellular destinations are met. Various genes involved in tightly regulating the cell cycle in ovarian cancer. A: Deregulation of the key genes (black circle with a star) initiates aberrant proliferation triggering the development of neoplasms. Single or multiple pathways may be involved in the process. In early stage development there are chances of single gene mutations or epigenetic mechanisms that transform the cell into an immortal phenotype. However, when multiple pathways are hit or crucial genes such as NF-kB are mutated, far more aggressive tumors can develop. Symbols used: open circle, normal cell; open oval, cell towards senescence or apoptosis; black jagged star, cancerous cell; black circle with white star, mutated gene/pathway; black circle with P, phosphorylated. B: Gradual stages of early to late ovarian tumor development and pathway abnormalities have been highlighted in miniature figures below the main one. Symbol used: gray cloud-like structure, tumor.

Of the many regulators, tumor suppressor proteins play a pivotal role in the cell cycle process and these proteins are highly deregulated in ovarian cancers (Table 1).

Tumor suppressor genes, proto-oncogenes and cell-cycle proteins cohesively regulate cell growth and division. The primary cyclins and kinase inhibitors that have a major role in ovarian cancer are cyclin D1, p16INK4a, p27, and p21 (Table 1, Fig. 2; Vikhanskaya et al., 1996; Kusume et al., 1999; Shigemasa et al., 1999; Bali et al., 2004; Barbieri et al., 2004; Hashimoto et al., 2011). The regulation and function of cyclin D1, p16INK4a, p27, and p21 have been shown to be controlled in part by the tumor suppressor gene p53, which is predominantly mutated in high grade ovarian cancers (Shigemasa et al., 1999; Bali et al., 2004). The changes in p53 as seen in ovarian cancers could be both at the gene and protein level. Point mutations, missense mutations and truncations have been observed, and overexpression of the p53 protein has been detected in many of the immunohistochemical studies that have been performed (Table 1; Marks et al., 1991; Liu et al., 1994; Havrilesky et al., 2003). The accumulation of non-functional p53 has been shown to affect the expression and interaction of proapoptotic genes such as
BCL2-associated X protein (BAX) and B-cell lymphoma 2 (BCL-2; Tai et al., 1998; Schuyer et al., 2001; Ziolkowska-Seta et al., 2009). The aberrations in p53 result in the accumulation of the altered protein within the cell that has a negative effect on BAX, a transcriptional target of p53 (Ozer et al., 2012). It seems likely that p53 alterations in high grade tumors reduce BAX expression allowing the progression of solid tumors. However, immunohistological studies have not provided a definitive conclusion of a positive correlation with p53 expression and tumor prognosis and survival (Reles et al., 2001;
Rose et al., 2003; Leffers et al., 2008). Contradictions in observations are possible due to the type of ovarian tissue analyzed, the method used and statistical programs employed to determine the correlation (Table 1).

The BCL family of apoptotic proteins along with p53 can serve as tools for histotyping (Skirnisdottir et al., 2002). The expression of p53-BCL-2 and BCL-2-BAX have been shown to have a strong association with tumor grade and histopathological subtyping, factors that could be vital for identifying the specific EOC for adjuvant or combined therapies (Skirnisdottir et al., 2002). What has been observed is that ovarian tumors are initially very responsive to treatment but later become chemo-resistant. Possibly the treatment itself may cause a few cells within the tumor mass to harbor mutations in p53 that, in addition to epigenetic silencing of promoter regions of apoptotic favorable genes such as p16 or Rb, may account for the relapse and progression of the tumor. Ovarian cancers are categorized as low or high-grade tumors of various histological subtypes (Le Page, 2010). These range from well-differentiated, to moderately differentiated to non-differentiated tumors, respectively, and morphologically are identified as benign, borderline or malignant ovarian tumors (Fig. 2; Le Page, 2010). Studies have shown that in low-grade ovarian tumors, p53 mutations are absent, but transitioning into the aggressive type, a significant increase in mutations of the gene are observed, with a majority of them being of the serous epithelial type (Marks et al., 1991; Kohler et al., 1993). The factors that contribute to this observed increase in p53 mutations are still very unclear. p53 is a quintessential player in the cell cycle and its role in maintaining healthy cell populations is very apparent. Since ovarian cancers are of multi-histological phenotype the alterations of p53 in tumors of this type may be varied. An
altered state of p53 is observed both at the gene and at the protein level in type 2 ovarian
cancers (Yemelyanova et al., 2011; Jones et al., 2012; Kuhn et al., 2012). Most mutations
observed in p53 gene are point mutations that cause the accumulation of the non-
functional protein in the cell due to increased stability. In some instances, the binding
domains of p53 could be altered such that p53 cannot bind to the DNA binding elements
of its target genes hindering subsequent pathways that encourage apoptosis. In other
scenarios, the functional role of the non-functional p53 is based on p16INK4a, a
cyclin kinase inhibitor within the cell (Leong et al., 2009).

**p21 and p27**
Cyclin dependent kinase (CDK) inhibitors are major coregulatory proteins in the cell
cycle along with the p16, p53 and retinoblastoma (Rb) pathways (Sherr, 1996; Bartkova
et al., 2003; Jayasurya et al., 2004). p21 is a direct transcriptional target of p53 (Gallagher
et al., 2012). In the presence of wildtype (WT) p53, p21 induction ensues followed by the
inhibition of cyclin E/CDK2 preventing the G1-S transition, encouraging the apoptotic
phenotype of cells (Hindley and Philpott, 2012). CDK inhibitors p21 and p27 control
various phases of the cell cycle based on the cyclin with which they associate. p21
association with cyclin D-CDK4/6 inhibitsG1-S transition; with cyclin E-CDK2 the late
G1-S transition; with cyclin A-CDK2 preventing the S-G2 transition; and with cyclinA-
cdc2 the G2-M transition (Cariou et al., 2000; Sandhu et al., 2000). p27 exhibits the same
level of control with cyclins D, E and A from the G1 to the G2 phase (Cariou et al.,
2000). Although the roles of p21 and p27 have been discussed in many cancers, they
appear to have secondary roles in the etiology of ovarian cancer rather than in the genesis (Milde-Langosch et al., 2003; Plisiecka-Halasa et al., 2003; Oudit et al., 2004; Lee et al., 2007). The role of p27 in ovarian cancer is somewhat contradictory. On examination of the protein level and localization of p27 in 150 advanced epithelium ovarian cancers (EOCs), lower nuclear p27 expression was associated with an improved prognosis (Psyrri et al., 2005). However, another study found that negative p27 expression was associated with poor prognosis (Shigemasa et al., 2001). Possibly, different grading scales, techniques and samples assessed might account for these inconsistencies. Loss of function or accumulation of these proteins in ovarian cancers has not been reported. In some cases, the absence of or low expression of p21 with p53 expression appears to place patients at a higher risk for disease recurrence (Anttila et al., 1999b). Studies have also shown that high p21 expression correlates with early stage ovarian tumors and that only high p27 expression was necessary for disease free survival (Schmider-Ross et al., 2006). Overall, there appears to be no correlation between p21 and p27 expression and in terms of ovarian cancers, as yet these proteins are not especially useful tools for the prognosis of the disease (Baekelandt et al., 1999).

Pathways Implicated in the Biogenesis of Ovarian Cancer

p16INK4a and Rb pathway

In normal cells, p16INK4a regulates cell proliferation by promoting genes of the apoptotic pathways (Leong et al., 2009). However, in ovarian cancer cells, it has been observed that p16INK4a is mutated or its promoter region is hypermethylated switching off its expression. Transfection studies using ovarian cancer cell lines have shown that in
the absence of WT p53, p16INK4a is upregulated to rescue the apoptotic pathway (Modesitt et al., 2001; Ramirez et al., 2001). In cells that contain double defects of both p53 and p16INK4a, cell proliferation is rampant and the tumors are more aggressive (Modesitt et al., 2001; Ramirez et al., 2001). Far advanced tumors have been shown to have low expression of p16INK4a but higher expressions of retinoblastoma (Rb; Todd et al., 2000). p16INK4a is considered to be a direct target of Rb expression (Todd et al., 2000). However, some studies have contradicted this finding. It appears that p16INK4a and Rb mutually regulate the expression of one another. While trying to understand the mechanistic pathways involved in ovarian cancers, the Rb-p16INK4a pathway in conjunction with p53 has been implicated in the genesis of a majority of cancers and has a pivotal role in the ovarian epithelial specific type (Konecny et al., 2011). The p16INK4a inhibitor of kinase prevents the association of the cyclin D1-CDK4/6 complex necessary for the transition of cells from the G1-S phase, in conjunction with hyperphosphorylated Rb. Studies performed to determine the expression patterns of these keys genes have shown that loss of p16INK4a function through promoter methylation is more frequent than mutations and deletions, in comparison to Rb, where hemizygous deletions are more pronounced with few mutations (Todd et al., 2000; Hashiguchi et al., 2001). The level of cyclin D1 necessary for cyclin-CDK complex formation is overexpressed and has been found to be associated with aggressive phenotype and poor prognosis in about 19% of ovarian cancers. (Todd et al., 2000; Konecny et al., 2011). As discussed, although alterations/loss of function appear to be the highlighted changes associated in the Rb-p16INK4a pathway, most ovarian cancers appear to co-express Rb and p16 proteins, with overexpression of Rb found in advanced ovarian tumors (Todd et
Post-translational modifications that affect protein function are also an important factor to consider in the analysis of tumor biogenesis. The Rb protein release from the Rb-E2F complex occurs by the hyperphosphorylation of its serine/threonine residues. A further examination of the phosphorylation levels of Rb protein therefore needs to be examined. Post-translational modifications have been found to affect protein function based on the residues phosphorylated as seen in p53 (Smeenk et al., 2011). In addition, epigenetic alterations as seen in the p16INK4a gene, may also regulate Rb and p53 function through CCCTC binding factor (CTCF; De La Rosa-Velazquez et al., 2007; Soto-Reyes and Recillas-Targa, 2010).

**KRAS/MAPK/ERK pathway**

Kirsten rat sarcoma oncogene (KRAS) activation triggers a sequence of events through the RAF/MEK and mitogen activated protein kinases (MAPK) pathways, and in conjunction with mammalian target of rapamycin (mTOR), a target of the protein AK strain thymoma (AKT) pathway, control cell proliferation (Table 2; Janku et al., 2012). Point mutations in KRAS provide an advantage for the survival and progression of tumors (Mane et al., 1990; Edkins et al., 2006; Harris and McCormick, 2010; Li et al., 2011; Oliveira-Cunha et al., 2012). KRAS mutations have been implicated in the genesis of lowgrade ovarian tumors, inducing an overactive proliferative phenotype (Vereczkey et al., 2011; Stewart et al., 2012). In addition to breast cancer associated protein 1 (BRCA1) and 2 (BRCA2) that have familial roles in sporadic germline-based breast and ovarian cancers, KRAS is now considered the third player (Kundu et al., 2012; Pilarski et al., 2012). KRAS expression levels differ based on the histopathological type
of ovarian tissue and the expression levels may help determine the various type of ovarian epithelial cancers (Auner et al., 2009; Steffensen et al., 2011b; Nowak-Markwitz and Spaczynski, 2012). These signatures have a specific pattern in tandem with the expression of RAF/MAPK components, phosphatase and tensin homolog (PTEN) levels and AKT (Table 2) and may help differentiate normal from borderline to early-stage cancers. Determining the signatorial patterns is relatively difficult due to heterogeneity of the tumors.

A mutant form of KRAS, called KRAS-variant, carries a mutation in its sequence that has the binding site of a micro RNA (miRNA) termed lethal-7 (Let-7), limiting the association of Let-7 with KRAS (Ratner et al., 2010). Consequently the levels of KRAS rises followed by the induction of the nuclear transcription factor (NF-kB) that promotes the induction of anti-apoptotic genes. It is quite probable that in late stage ovarian cancers, the earlier mutations in KRAS can stimulate and induce the over-activation of NF-kB in cancer stem cells (CSCs) that survive chemotherapy. However, immunohistochemical-based correlation assessments between the KRAS expression and alterations in NF-kB components and PTEN have not yet been able to confirm a direct association (Laudanski et al., 2011). KRAS-variants have also been shown to target NF-kB expression independent of the phosphatidylinositol-3 kinase PI3K-AKT pathway.
(Keane and Ratner, 2010; Ratner et al., 2010, 2012; Kinross et al., 2011; Mizumoto et al., 2011; Pharoah et al., 2011; Weidhaas and Slack, 2011; Pilarski et al., 2012). The disparities in KRAS expression and association patterns with downstream targets may stem from the type of tissues analyzed. In a study conducted on 489 high grade ovarian adenocarcinomas, Spellman et al. (2011) showed that in 45% cases that contained altered PI3K/RAS signaling, less than 1% was due to mutations and 11% were amplifications. Also, the downstream target, serine/threonine-protein kinase B-Raf (BRAF) was mutated (0.5%). In addition to PI3K/RAS signaling, KRAS induces mitogen activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) through BRAF. Stimulation of KRAS via GTPase activity activates BRAF. The stimulation can be mediated by cytokines, growth factors or proto-oncogenes. The downstream target of BRAF, MEK stimulates ERK when activated. ERK targets transcription factors that are involved in cell proliferation such as Myc (Smolle et al., 2013). In ovarian carcinomas, activation of the ERK pathway stems from hormonal activation of G-protein coupled receptors (Smolle et al., 2013). MAPKs, part of the MAPK/ERK pathway, are involved in the transduction of signals through hormones. MAPKs are of two types, tyrosine protein kinase and G-protein coupled receptor. In ovarian cancers, hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) may participate in signal transduction via MAPKs, and are thought to be responsible for ovarian cancer cell growth in carcinomas that express the receptors (Hilliard et al., 2013).
PI3K/PTEN/AKT pathway

Phosphatidylinositide-3 kinase (PI3K) and PTEN proteins influence multiple pathways within the cell that have many effects on the cellular phenotype through protein AK strain thymoma (AKT), also known as protein kinase B (PKB). These genes are crucially required to transcend the input from external stimuli (growth factors) and convey them to AKT. Phosphorylation of key amino acid residues in these proteins triggers the downstream activation processes. PTEN is a tumor suppressor gene and either mutations in the gene or loss of function of the protein is observed in many cancers, including ovarian cancers (Table 2; Saga et al., 2002; Meng et al., 2006; Yan et al., 2006; Paige and Brown, 2008). Loss of function in this gene is mediated through epigenetic silencing and its role in ovarian cancer needs to be further assessed (Kurose et al., 2001). AKT is an oncogene, and its function and activation depends on the function of PTEN (Meng et al., 2006; Blanco-Aparicio et al., 2007). However, when PTEN becomes inactivated, or loss of function is observed, AKT is overexpressed which serves as an impetus for tumor formation by changes in sub-pathways such as NF-kB, mammalian target of rapamycin (mTOR), and p53, that in turn regulates proliferation, cell division, and cell apoptosis (Tables 1 and 2; Blanco-Aparicio et al., 2007; Hussain et al., 2012). PTEN and AKT are genes of a pathway controlled by PI3K phosphorylation of its internal amino acid residues, the signals of which depend on either growth receptors or tyrosine kinase receptors (Xu et al., 2004; Lee et al., 2005; Meng et al., 2006).

PI3K is a heterodimeric protein comprised of a regulatory subunit, p85, and a catalytic subunit, p110 (Berenjeno and Vanhaesebroeck, 2009; He et al., 2010; Folgiero et al., 2012; Hofmann and Jucker, 2012; Li et al., 2012; Takayama et al., 2012). The p110
catalytic subunit exits as isoforms a, b, g, and d (Li et al., 2012). This enzyme phosphorylates the hydroxyl group of inositol of phosphatidylinositol triggering endocellular pathways from external stimuli. For the activation of PI3K, autophosphorylation of tyrosine kinase receptors (RTKs) is essential (Smith et al., 2002). The p85 subunit of PI3K tethers to the phosphorylated residues of RTKs. Once the docking of its regulatory subunit occurs, p110 begins its phosphorylation of phosphatidylinositol-4,5 biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). (PIP3) is essential for the activation of AKT (Carnero, 2010). However, AKT activation requires phosphorylation activities of (PIP3) and mTOR (Carnero, 2010). PTEN, an intermediate factor of the PI3K/PTEN/AKT pathway, plays an important role in ensuring that the activity of AKT is held in check by dephosphorylating (PIP3) (Stocker et al., 2002). While assessing the roles of PI3K/PTEN/AKT in ovarian carcinogenesis, amplifications of PI3K, p110 a subunit, and loss of PTEN function are found (Blanco-Aparicio et al., 2007). When events such as these occur, AKT levels cannot be controlled triggering pathways of survival, progression and invasion through subpathways controlled by AKT (Blanco-Aparicio et al., 2007). It has been observed that inhibition of p110 a subunit or AKT, can induce cell cycle regulatory proteins, p21 and p27, inhibit phosphorylation of Rb and reduce the levels of cyclins D1 and CDK4 that essentially serve the apoptotic pathway (Meng et al., 2006). Some of the resistance offered by ovarian tumors to treatment by certain drugs stem from the amplification of PIK3Ca, a gene that encodes PI3K p110 a subunit, and AKT expression with reduced PTEN levels (Janku et al., 2011; Yamamoto et al., 2011; Abe et al., 2013). In such instances, cell cycle regulatory functions are impaired, and pro-apoptotic proteins such as
Bax translocation is inhibited (Lee et al., 2005). PI3K mutations in ovarian tumors are rare, but an increase in gene copy number has been observed. The amplifications are more pronounced in high grade ovarian tumors than low grade tumors along with AKT phosphorylation contributing to survival, and progression of the disease (Huang et al., 2011; Abe et al., 2013). There does not appear to be a collective association of the three proteins together in ovarian carcinogenesis and are thought to act independently (Carden et al., 2012). However, the alterations in the expression of these proteins have been shown to have a relationship between the resistance offered against therapies and the recurrence of the disease (Dent et al., 2009).

mTOR promotes cell growth and proliferation in association with the PI3K/AKT pathway (Dobbin and Landen, 2013). The mTOR has the ability to phosphorylate AKT at the serine residue 473 through its mammalian target of rapamycin complex 2 (mTORC2) complex. The mammalian target of rapamycin complex 1 (mTORC1), raptor, and mTORC2, rictor, differ in composition by a few elements that make up the respective complexes, in addition to difference in resistance to rapamycin (Mabuchi et al., 2011). mTORC1 promotes cell growth and cell mass by the activation of molecules involved in protein synthesis (No et al., 2011). mTORC2 promotes cell survival and proliferation through AKT activation. Under normal physiological conditions in the ovary, the PI3K/AKT/mTOR pathway protects the primordial follicles from destruction. However, in the presence of environmental toxins, overstimulation of the pathway results in follicular proliferation, depletion of primordial follicles and an induction of a condition called premature ovarian failure (Borman et al., 2000; Sobinoff et al., 2011). Together, PI3K/ AKT/mTOR contributes to cell survival, and proliferation. The PI3K/AKT/mTOR
pathway is involved in type I and type II ovarian cancers. Single mutations of a member of the pathway coupled with a mutation with members of another pathway promote ovarian hyperplasia, and double mutations within the same pathway are necessary for ovarian tumorigenesis. Mutations in parallel pathways that are involved in cross-talk are found to be mutated in ovarian carcinomas. The integrated genomic analysis study of ovarian carcinomas showed that at least 45% of the cases contained mutations in the PI3K/RAS signaling pathway, where PTEN deletions (7%); mutations (<1%), PIK3CA amplifications (18%); mutations (<1%), AKT isoform amplifications AKT 1 and AKT 2 (3 and 6%, respectively), were observed in conjunction with KRAS amplification (11%; Spellman et al., 2011). KRAS independently controls survival through BRAF but can also activate PIK3CA that induces AKT and cell cycle progression (Spellman et al., 2011). Similarly, in the PI3K/AKT/mTOR signaling, a study by Kinross et al. (2011) using a mouse model demonstrated that PTEN double deletions with PIK3CA activation was necessary for ovarian serous adenocarcinomas and granulosa cell tumors (Dobbin and Landen, 2013). Therefore, a single event does not appear to influence ovarian tumorigenesis but multiple hits in the pathways that regulate growth, proliferation, and survival are required for tumorigenesis and progression. Thus, a one cure all for ovarian tumors cannot exist. Rather, ovarian tumor type-pathway specific form of treatment may be more appropriate.

**Hedgehog pathway**

Hedgehog is a signaling pathway that controls development and is expressed during embryonal development (Chen et al., 2007). Adult tissues have significantly reduced
expression of the protein and alterations, either by mutations of mediators of the pathway or overexpression of the ligand receptor, appear to promote ovarian tumorigenesis (Chen et al., 2013a). Sonic hedgehog (SHh), the Indian hedgehog (IHh), and the dessert hedgehog (DHh) have distinct biological roles and induce effects through 10-pass transmembrane patch (Ptch; Chen et al., 2013a). The canonical stimulation of Hh pathway induces the 7-pass transmembrane smoothend (Smo)-triggered Gli activation of Hh targeted genes through Ptch inhibition (Chen et al., 2013a). The hedgehog signaling pathway is important to organ development at early embryonal stages and is selectively expressed during tissue maintenance and repair and in stem cells (Song et al., 2011; Coni et al., 2013). The pathway can be activated by ligand dependent or independent mechanisms (Ehteshamet al., 2007; Christiansen et al., 2012). Overexpression of the ligand receptor or mutations of the members in the pathway can contribute to cancer progression. Activation through epidermal growth factor receptor (EGFR) constitute the noncanonical mode of Hh signaling (Mangelberger et al., 2012). The expression of Hh in adult ovarian tissue has not been observed. However, the signaling mechanism is likely to be activated within the stem cell population in the ovarian tissue that is necessary for the repair of the ovarian surface epithelium (OSE). Cancers arising from OSE have an epithelial phenotype. They are thought to arise through mutated Hh signaling and produce spheroid like structures with cancer stem cell-like properties (Ray et al., 2011). Suppressor of fused (Su(fu)), a tumor suppressor, is a repressor of the Hh pathway and loss of Su(fu) accounts for increased Hh signaling (Cheng and Yue, 2008). Reports suggest that Hh helps with clonal growth, and that the expression of members of the pathway, Gli1 and patched correlate with poor outcome. The three ligands of Hh
signaling have distinct biological functions and yet, it appears that of the three ligands, SHh is the prominent ligand-overexpressed. It remains to be ascertained if the ligand-type overexpressed is ovarian-tumor-type specific or if the overexpression of any one ligand suffices to induce an ovarian tumor phenotype. Studies have shown that using SHh and IHh agonists, the number of spheroid formations increase (Ray et al., 2011). SHh expression has also been found to be expressed in ovarian dermoid and requires the induction of Gli (Sabol et al., 2012). DHh has been shown to correlate with poor prognosis (Chen et al., 2013a). Finally, the role of Hh pathway dysfunction in ovarian carcinogenesis is still not completely understood as its association with other signaling pathways in addition to its non-canonical pathway adds to the complexity.

The Wnt pathway is important to ovarian follicular development (Sarkar et al., 2010). The Wnt pathway is activated downstream of Hh pathway and Gli transcription factors, terminal activators of Hh, induce Wnt ligands. Also, glycogen synthase kinase-3B (GSK-3B) of Wnt pathway regulates molecules of Hh signaling (Sarkar et al., 2010). The Wnt/B-catenin, the canonical pathway of Wnt signaling influences oncogenic Hh signaling. Thus cross-talk activity between the two pathways is apparent. An analysis of 26 and 20 genes of the Hh and Wnt pathway, respectively, of matched fresh frozen and paraffin embedded ovarian endometrial carcinomas showed that the expression of tumor-specific genes associated with Hh and Wnt pathway were not consistent and those that showed statistical significance had varied genetic profiles (Steg et al., 2006). Overexpression of genes SMO, GLI, GLI2, GLI3, and Wnt7A, Frizzled homolog (FRZD1), low-density lipoprotein receptor-related protein 6 (LRP6), and Frizzled related
protein (FRZB) that was detected in both freshly frozen and FFPE tissue, were not statistically significant. Similar observations was seen with genes DHH, IHH, SHH, PTCH, PTCH2, GLI, GLI3, and SMO specific to the Hh pathway (Steg et al., 2006). For suitable anti-pathway therapies, further studies that determine specific change from inter-individual variations are warranted. From a treatment perspective, an ovarian tumor type-pathway specific form of treatment is therefore more appropriate.

Multiple drug resistant (MDR) pathway

ATP-binding cassette (ABC) transporters are implicated in multidrug resistance (MDR) of many tumors. Ovarian cancers by far appear to build up resistance to many treatments and ABC upregulation is thought to play a major role in this process (Januchowski et al., 2013). A comparative study analyzing benign versus recurrent ovarian tumors showed that of the 9 ABC transporters, 4 (ABCC1, ABCC2, ABCC3, and ABCB3) of them are significantly elevated in recurrent ovarian tumors (Auner et al., 2010). Analysis of these four transporters in recurrent versus primary ovarian lesions transporters showed significant differences but were minor and insignificant with respect to benign tumors. Changes in ABC transporters appear to stem from treatment rather than in situ tumor development. Abnormal expression of the transcription factor Gli1, a downstream target of Hh signaling, has been shown to induce MDR resistance in a subset of ovarian cancers and that the promoter regions of ABCB1 and ABCG2 genes contain Gli1 binding specific consensus sequence (Sims-Mourtada et al., 2007; Chen et al., 2013b).
**Notch pathway**

Activation of Notch signaling pathway is important to cell fate determination and organogenesis in embryogenesis (Reynaud-Deonauth et al., 2002; Petersen et al., 2006). Downstream targets of Notch signaling such as hairy and enhancer of split-1 (HES1) are expressed in many ovarian cancers indicative of the role of Notch (Schreck et al., 2010; Wang et al., 2010a). Notch pathway is regulated by ligands such as Jagged1, 2 and Delta-like 1, 3, 4 (Fleming et al., 1997; Lendahl, 1998; Gray et al., 1999; Yamaguchi et al., 2002). When Notch receptors are activated by ligand binding, the transmembrane portion of the receptor undergoes postranslational modifications and a complex cleavage process (Pratt et al., 2011). The cleaved C-terminal domain then translocates to the nucleus and induces downstream targets such as HES1, a transcriptional factor (Schreck et al., 2010). Elements of the Notch pathway are expressed in EOCs (Hopfer et al., 2005). Notch pathway signaling appears to be fundamentally important to cell survival, motility and development of vasculature (Shin et al., 2008; Li et al., 2010). The chemoresistance observed to platinum-based therapy of ovarian cancers stems from the expansion of cancer stem cells that have Notch activated (Notch 3) (McAuliffe et al., 2012). A study analyzing pathway deregulations in ovarian tissue specimens showed that of the tumors analyzed, 22% had altered Notch signaling, mostly as amplifications in the ligand Jagged 1 and 2 and Notch 3 (Spellman et al., 2011). Overactivation of Notch ligands (Jagged 1 and 2) has been shown to be mediated through the stimulation of p73, a member of p53 (Sasaki et al., 2002).
Forkhead box M1 pathway (FOXM1)

FOXM1 is a transcription factor that regulates genes that control the cell cycle and thus proliferation and tumor progression (Mencalha et al., 2012; Yang et al., 2013) and its role in angiogenesis has also been observed (Li et al., 2009). Serous ovarian cancers express high levels of the protein that correlates with tumor progression (Lok et al., 2011). In the study by Spellman et al. (2011), 85% of the cases studied showed alterations in FOXM1 pathway. Of the important elements of the FOXM1 pathway, baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5/survivin) and cell cyclin B1 (CCNB1) were found to be highly up-regulated when compared to aurora kinase-B (AURKB), cell division cycle 25 homolog B (CDC25B) and polo-like kinase 1 (PLK1) (Spellman et al., 2011). Collectively, the proteins are required for regulated cell cycle progression and aberrations lead to deregulated tumor progression. FOXM1 is also involved in the induction of breast cancer type 2 susceptibility protein (BRCA2) a downstream target that regulates DNA repair (Kwok et al., 2010; Millour et al., 2011). FOXM1 is involved in cell migration/invasion in ovarian cancers via ERK that acts upstream of FOXM1 (Lok et al., 2011) and regulates cell proliferation through a number of elements in the pathway.

Breast Cancer Type 1/2 Susceptibility Protein (BRCA1)/(BRCA2) and Homologous Repair and Nucleotide Excision Repair Pathway

BRCA1 and BRCA 2 are tumor suppressors that readily associate with p53 to serve apoptotic functions (Zhang et al., 1998; Abramovitch and Werner, 2003; Navaraj
et al., 2009). Most of the mutations seen in ovarian tumors are somatic mutations (Zweemer et al., 1999; Amikura et al., 2006; Usha et al., 2011; Szabova et al., 2012). However, mutations in BRCA1 and BRCA2 are heritable germline mutations. Only 10% of the ovarian tumors that arise are heritable, which involve the BRCA1/2 mutations (Zhou et al., 2013). The remaining 90% arise through somatic mutations affecting proteins such as BRCA1/2 or p53 (Zikan et al., 2008). A study conducted by Zweemer et al. (1999) determined the relationship between p53 accumulation and the presence of BRCA1, BRCA2 or both mutations, respectively. When compared alone to the specific mutation itself, it was found that p53 accumulation was more pronounced with BRCA1 than BRCA2 mutation (Zweemer et al., 1999). However, similar observations are seen in the absence of BRCA mutations (Cramer, 2012). Thus the correlation between p53 and BRCA mutated genes need further clarification. BRCA1 expression is favored over BRCA2 expression in terms of staging the cancer. p53 accumulation is more apparent with BRCA1 mutations in late stage or stage III ovarian cancers (Johannsson et al., 1997; Kaern et al., 2005; Wysham et al., 2012).

BRCA proteins are associated with DNA repair and exist as a complex with other repair proteins (Brugarolas and Jacks, 1997; Jasin, 2002; Tutt and Ashworth, 2002). Hereditable ovarian cancer involving BRCA requires two-hits for tumor formation. A single BRCA1 affected allele may not be sufficient to promote tumor formation. However, DNA stability is affected as DNA repair is affected that can encourage tumor formation through the loss of function of the second allele or mutations in genes governing the repair pathway. The paradoxical role of BRCA in cancers is apparent (Powell and Kachnic, 2003). Hereditable and somatic mutations in BRCA are responsible
for breast and ovarian cancer formation, and yet, cells that carry WT BRCA with other pathway anomalies are less sensitive to treatment or develop chemoresistance. The observations are controversial as some studies point out that the BRCA status (proficient vs. absent) of the cells do not have a significant correlation to treatment outcome, whereas others have shown that the absence of BRCA1 enhances the sensitivity to treatment by agents that induce DNA damage, including ionizing radiations (Wiltshire et al., 2007; Johnson et al., 2011). Spellman et al. (2011) showed that the presence of BRCA1 mutations was associated with better survival outcomes as compared to those patients that carried WT BRCA1.

Current therapies against ovarian cancer involve cisplatin treatment (Laios et al., 2013; Song et al., 2013). Patients that are initially responsive become resistant to the treatment. Platinum-based cisplatin therapy involves the induction of DNA damage through inter and intrastrand crosslinking between purines (Basu and Krishnamurthy, 2010). The 1, 3 and the 1, 2-intrastrand crosslinks are excised and removed through nucleotide excision repair (NER) with the former lesions being easier to remove due to less distortion of the helix (Enoiu et al., 2012). The 1, 3-interstrand lesion are complex and require homologous recombination where double stranded breaks (DSB) are involved (Hinz, 2010). The NER system is robust in terms of lesion recognition and requires a host of various NER components. The up-regulation of these elements by DNA-induced damage could account for the gain of resistance to treatment. In terms of DSBs, Rad51 recombinase is required and acts in conjunction with BRCA2 (Davies et al., 2001). Therefore, loss of function of BRCA2 or mutations that silence the expression of the protein favors sensitivity to treatment and thus women with BRCA1/2 mutations have a
better diagnosis and are more responsive to platinum-based therapy. The direct correlation between the expression of NER genes or its members and resistance to therapy does not always hold true. The data obtained from a study analyzing NER efficiency and cisplatin resistance showed that altering the HRR pathway, but not NER member expression, could enhance the sensitivity of cisplatin-resistant tumors to platinum-based agents (Wang et al., 2011). These observations have been corroborated by the Spellman et al. study that showed that of the samples tested 23% carried mutations/epigenetic altered states in BRCA1, while 11% carried mutations in BRCA2 (Spellman et al., 2011) and that 51% of the cases had altered HRR pathway that involved BRCA2 and Rad51C (Spellman et al., 2011).

**Epigenetics and ovarian cancer**

Histones and DNA are primary targets of epigenetic regulation (Seeber and van Diest, 2012). Genes that harbor CpG islands are susceptible to epigenetic modifications, consisting primarily of DNA methylation (Maradeo and Cairns, 2011). Both types, histone-based and DNA-based methylation marks, are largely known to inhibit gene expression through transcriptional inhibition (Seeber and van Diest, 2012). Of the amino acid residues, lysines are more prone to epigenetic modifications via methylation, acetylation, phosphorylation sumoylation and ubiquitination (Saldanha and Tollefsbol, 2012). However, the well-studied acetylation modifications primarily affect the exposure of theDNAto the transcriptome machinery. In some cases, non-histone proteins like p53 when acetylated affect its cellular functions through its DNA binding interactions and
stability (Ito et al., 2002; Scoumanne and Chen, 2008; Pirola et al., 2012; Kim et al., 2013). Alterations to the epigenome have been shown to be instrumental in the genesis of diseased phenotypes. The current status of epigenomic research shows that DNA methylation, histone modifications, miRNA collectively alter epigenetic profiles of tissues that promote tumorigenesis. Epigenetic modifications are promising targets for treatment as the mechanisms can be reversed through epigenetic enzyme targeted therapy.

The role of methylation in cancer formation can be gene or loci specific. In general, hypermethylation of tumor suppressor genes and hypomethylation of oncogenes contribute to pathway deregulations that promote tumor formation. In the genesis of ovarian cancers, overexpression or silencing of members of the pathways that control proliferation and growth promote tumor initiation and progression. In mammalian germ cell-derived ovarian tumor, promotion is associated with the deregulation of pRb pathway where p16 reduction is observed. The reduction is found to be associated with the hypermethylation of p16 (INK4A) promoter region that correlates with cell promotion (Kawauchi et al., 2004). Increased methylation of tumor suppressor genes have also been observed in ovarian tumors (Chmelarova et al., 2012; Ozdemir et al., 2012). Increase in promoter methylation of O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT), paired box 5 (PAX5), Cadherin 13, H-Cadherin (Rose et al.) (CDH13), Wilms tumor 1 (WT1), Thrombospondin 1 (THBS1), and GATA5 have been observed in endometroid ovarian cancer as compared to serous ovarian cancer (Chmelarova et al., 2012). In the same study, surprisingly, commonly deregulated genes in ovarian cancer ataxia telangiectasia mutated (ATM), TP53, PTEN, Von Hippel–Lindau tumor
suppressor (VLH), glutathione S-transferase pi (GSTP1), RB1a, MGMTb, and PYCARD that encodes apoptosis-associated speck-like protein containing a CARD did not show significant methylation above the cut off value of 15% (Chmelarová et al., 2012). Another study showed that tumor suppressors cyclin-dependent kinase inhibitor 2B (CDKN2B), CDH13, and RASSF1, a gene that encodes Ras association domain-containing protein 1 have significant hypermethylation and that CDKN2B promoter hypermethylation was observed in clear cell carcinomas as compared to other histological types (Ozdemir et al., 2012). Hypermethylation of BRCA1 has been shown to be frequent in spontaneous breast and ovarian cancers (Wang et al., 2010b). Demethylation of BRCA1 appears to decrease chemosensitivity of platinum-sensitive cells associated with partial increase of BRCA1. Thus BRCA1 hypermethylation favors treatment sensitivity and has been shown to function independently of PI3K-Akt pathway (Wang et al., 2010b). Methylation analysis of ovarian tumors of genes involved in the Wnt pathway demonstrated that the naked cuticle homolog 1 (NKD1) and disheveled homolog (DVL1) methylation increased risk of disease progression (Dai et al., 2011). Hypermethylation of members of SHh pathway, zinc finger protein 1 (ZIC1), results in poor progression free survival (PFS; Huang et al., 2013). In addition to the role in PFS, silencing of SHh members, ZIC1 and zinc finger protein 4 (ZIC4) by methylation correlate with increased proliferation, migration, and invasion (Huang et al., 2013). Therefore, methylation shows promise as a marker for PFS. However, studies using larger sample size may be required to support the observation. The relationship between DNA hypermethylation generally favors reduced gene expression. The examination of 1,505 CpG sites between ovarian cancer cell lines and primary ovarian tumors showed
that ovarian cancer cell lines seem to exhibit distinct methylation profiles as compared to the primary ovarian tumors (Houshdaran et al., 2010). Ovarian cancer cell lines tend to have higher methylation patterns as compared to primary tumors. An explanation may be that ovarian derived cancer cell lines are pure cultures that represent one type of cell population. However, primary tumors are a heterogeneous mass of cells with a mixture of other cells as well. Therefore, careful interpretation is essential as to the use of methylation as a marker for PFS and also in terms of therapeutic treatments with various compounds that target methylation. These studies make it clear that preclinical findings cannot be directly applicable to in situ tumors. This may also account for the reason why so many treatments that work successfully in vitro fail to show promise in vivo.

Methylation of lysine residues takes many different forms; mono, bi and tri valencies (Zhang et al., 2012). However, the position and number of lysines methylated determine the methylation-based gene activity status. Bi- and tri-methylation of histone H3 lysine 27 (H3K27(me3)) contributes to gene silencing and tri-methylation of histone H3 lysine 4 residue (H3K4(me3)) activates gene expression (Payne and Braun, 2006; Lilja et al., 2013). In ovarian tumors, especially in the case of a subset of cells that escape chemotherapy, these epigenetic alterations are observed (Lotem and Sachs, 2006; Balch and Nephew, 2010; Min et al., 2012). What have been analyzed in studies thus far are comparisons of gene sets at various stages of ovarian cancer to normal tissue. The bivalent marks assessed in these studies are based on comparison of ovarian cancer stem cells to patterns in human embryonic stem cells (hES; Chapman-Rothe et al., 2012). These findings are important as chemoresistance and recurrence of more aggressive
tumors could stem from the population of cancer stem cells that have epigenetic plasticity. The process of ovulation and the stress exerted upon the organ due to ovulation requires the need of continual cell replacement. Continual change and plasticity of tissues are maintained by stem cells and studies have established the presence of such in the endometrium of uterine tissue (Teixeira et al., 2008). In all likelihood a similar scenario could exist in the ovary. Recent findings of the presence of adult stem cells in the ovaries is quite exciting and may provide a key link into how tumors arise in the ovary (Djordjevic et al., 2012; Foster et al., 2012). Linking the methylation patterns to these adult stem cells, normal epigenetic marks may undergo a change mediated by environmental cues (external/internal), for example, parity, inflammation, toward a more tumorigenic phenotype by the suppression or loss of tumor suppressor genes. Also, tumor suppressor genes that harbor these specific bi-and tri-methyl marks are more pronounced to gene silencing through epigenetics affecting pathways such as the PI3K pathway (Min et al., 2012; Seeber and van Diest, 2012).

In normal tissues, DNA hypomethylation of tumor suppressor genes is observed, which is reversed in tumorigenic tissues, where hypermethylation of CpG-promoter rich genes and global hypomethylation is predominant (Bammidi et al., 2012; Ozdemir et al., 2012). The patterns can be readily reversed by targeting enzymes that regulate these transient modifications. DNA methyltransferases (DNMTs), histone acetyl transferases (HATs) and histone deacetylases (HDACs) serve as targets for therapies against many cancers (Cherblanc et al., 2012) However, single therapies have been found to be less effective against solid tumors as compared to combined therapy of conventional drugs with epigenetic therapies or combined epigenetic therapies.
There is a possible cross talk between DNA methylation and histone modification that dictates the dynamic states of chromatin and the genes associated with tumor biology. Hypoacetylation of H3 and H4 in association with GATA4 and 6 transcription factors have been found in a variety of ovarian cancer cells (Caslini et al., 2006). Methylation patterns such as trimethylation of H3K27 or dimethylation of H3K4 have been found to exist in carcinogenic ovarian tissue (Marsit et al., 2006; Chapman-Rothe et al., 2012). Hypermethylation of tumor suppressor genes such as PTEN and p16INK4a have been observed contributing to the loss of function of these proteins in EOCs (Yang et al., 2006; Tam et al., 2007) and the absence of these proteins contribute to deregulated pathways that have been discussed earlier in the review.

Micro RNAs (miRNAs) are tightly controlled in normal cells but become highly deregulated in cancer cells. They are single stranded non-coding RNA molecules about 22 nucleotides in length and regulate the levels of gene expression by performing silencer-like type functions, degrading themRNA to which they bind (Kuhlmann et al., 2012). They bind either to certain sequences within the mRNA or to the 3’-untranslated region of the gene. The role of some of the miRNAs in the etiology of various cancers have been well established (Kuhlmann et al., 2012). A detailed review of miRNAs and their role in ovarian cancer has been discussed in detail in Chen et al. (2011). The posttranscriptional modification of genes by miRNA and the presence of varied miRNA expression levels within solid tumors provides a map of miRNA signatures for specific cancers (Baer et al., 2013). Formulating drugs against these miRNAs may provide for a therapeutic approach. Table 3 lists the various miRNAs that play a significant role in ovarian cancers and the targets they affect in the process. For example, p27 is a cell cycle
regulatory protein whose post-transcriptional level is altered by the deregulated expression of miRNA 221 and miRNA 222 in ovarian cancers. They inhibit the expression of p27 that is essential to the control of cellular apoptosis thereby promoting cell proliferation (le Sage et al., 2007).

Epigenetic marks may prove useful in the diagnosis, prognosis and prediction of the disease. In the early stages of ovarian cancer, individuals are responsive to the treatment but eventually become chemoresistant, and the presence of cancer stem cells (CSCs) in the tumor mass may be responsible for the observed chemoresistance. Altering the epigenome of these CSCs may prove to be an alternate approach to targeting advanced ovarian cancers and re-sensitizing cells to chemical treatments and regression of tumors.

**Future Directions**

Ovarian cancers are lethal diseases as they slip detection and are far advanced when detected. Research in ovarian cancer has just scratched the surface in terms of understanding the pathways deregulated in the disease. Clearly, there does not appear to be a strong association between deregulated patterns and the gene specific expression and subcellular correlation patterns. Thus, treatment approaches are still not very effective.
This may be due to the fact that most preclinical studies of the disease have been based on the immunohistochemistry of the tissue. Utilizing more quantitative technologies such as microarray systems, western blots, real-time PCR, or whole-genome sequence analysis might provide different insights into the etiology and pathology of the disease. The information generated from these technologies can provide a wealth of information that relates to the mitotic and apoptotic deregulations of cellular pathways, histone signatures, DNA methylation patterns and miRNA expression patterns governing gene expression in ovarian cancer and its subtypes making treatments and therapies more customized.

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