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Epigenetics/epigenomics and prevention of early stages of cancer by isothiocyanates Review

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Abstract

Cancer is a complex disease and cancer development takes 10–50 years involving epigenetics. Evidence suggests that ~80% of human cancers are linked to environmental factors impinging upon genetics/epigenetics. Since advanced metastasized cancers are resistant to radiation/chemotherapeutic drugs, cancer prevention by relatively non-toxic chemopreventive “epigenetic modifiers” involving epigenetics/epigenomics is logical. Isothiocyanates are relatively non-toxic at low nutritional and even higher pharmacological doses, with good oral bioavailability, potent anti-oxidative stress/anti-inflammatory activities, possess epigenetic modifying properties, great anti-cancer efficacy in many *in vitro* cell culture and *in vivo* animal models. This review summarizes the latest advances on the role of epigenetics/epigenomics by isothiocyanates in prevention of skin, colon, lung, breast, and prostate cancers. The exact molecular mechanism how isothiocyanates modify the epigenetic/epigenomic machinery is unclear. We postulate “redox” processes would play important roles. Additionally, isothiocyanates sulforaphane and phenethyl-isothiocyanate, possess multifaceted molecular mechanisms would be considered as “general” cancer preventive agents not unlike chemotherapeutic agents like platinum-based or taxane-based drugs. Analogous to chemotherapeutic agents, the isothiocyanates would need to be used in combination with other non-toxic chemopreventive phytochemicals or drugs such as NSAIDs, 5- α -reductase/aromatase inhibitors targeting different signaling pathways would be logical for the prevention of progression of tumors to late advanced metastatic states.

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Introduction

Cancer is a complex chronic disease and cancer development is a multistep process (1, 2). In a simplified manner, it would involve initiation, promotion, to progression/metastasis (3, 4). Recent evidence suggests that many chronic illnesses including cancer are driven by epigenetics/epigenomics caused by environmental factors impinging upon the underlying genetic information (4–7). Since advanced metastasized cancer are resistant to radiation and chemotherapeutic drugs, prevention of early stages of cancer by relatively non-toxic dietary phytochemicals would be logical.

Role of epigenetics/epigenomics during the “long process” of cancer development

During the long process of cancer development particularly during the “promotion” stage, epigenetics has been postulated to play an important role in driving cellular transformation such as stem cells in forming benign microscopic tumor (8). Feinberg et al. has shown since the 1980s that most if not all tumors could be associated with widespread losses and some gains of DNA methylation throughout the genome (9, 10). This is reviewed recently in (6, 11), and elaborated on the role of epigenetics during cancer development. However it is still unclear how epigenetics would be integrated with the different hallmarks of cancer as discussed by Weinberg et al. (12).

Epigenetics or in a more global context epigenomics is a very complex process involving modulation of epigenetic modifiers including the ‘readers’, ‘writers’ and ‘erasers’ with involvement of multiple events including methylation, acetylation, ubiquitination. (13). Accumulating evidence also suggests that multiple genetic and epigenetic alterations would occur concurrently during tumor development (6, 14). In this context, the epigenetic marks (DNA methylation, histone modifications, chromatin remodeling, and non-coding RNA) could be used as potential markers of different stages of cancer development including initiation, progression, and metastasis (15, 16).

From our recent UVB-induced skin carcinogenesis study, global CpG methylation changes more dramatically in early stages of 2 weeks as compared to 15 weeks and 25 weeks post UVB irradiation. The CpG methylome changes decreased as time progresses taken into account of the effect of aging (17). Imaging assisted evaluation of microscopic tumors may have suggested that during the early promotion stage of carcinogenesis, drastic epigenomic aberrations occurs particularly with the progenitor stem cells driving phenotypic gene expression, may occur before mutational events (6). These epigenomic alterations would involve stem cell dysregulation blocking differentiation, dysregulation of multiple pathways including DNA repair and cell cycle, apoptosis/autophagy, cellular defense, and

inflammatory pathways. However, the underlying mechanism of UVB-induced global epigenomic changes in the skin is unclear. Acutely, UVB triggers oxidative stress, inflammation and DNA damage (18, 19), would be postulated to be some of the major drivers of epigenetic alterations.

Analyzing primary cutaneous melanoma samples for their global DNA methylation, found more than 98% loss of methylation and about 2% gain of methylation (20). *In vitro* study using melanoma cells showed that histone hypoacetylation is associated with downregulation of certain pro-apoptotic proteins, including Bak, Bim, and Bax, which belong to the BCL-2 family (21). Epigenetic reprogramming appears to play an increasing role in drug resistant in melanoma (22). In skin transformation model using epidermal JB6 cells, when JB6 cells were challenged with tumor promoter TPA (12-O-Tetradecanoylphorbol-13-acetate), significant alterations of DNA methylome coupled with transcriptome (23). In a recent UVB-induced mouse carcinogenesis epigenomic study, about 60% of the DNA-methylated-regions (DMRs) showed inversed relationship between DNA methylation and RNA expression, i.e., hypermethylation coupled with suppression of transcription or hypomethylation coupled with promotion of transcription, while the other 40% of these DMRs did not show such relationship (23).

In breast cancer, many studies have shown strong association of aberrant DNA hypomethylation with cancer development (24). Histone modifications such as loss of H4K16 acetylation has been implicated as an early event in breast cancer development and is associated with altered level of NAD-dependent histone deacetylases (HDACs); SIRT1 (25, 26).

In prostate cancer, up-regulation of EZH2 (Histone Methyltransferase, Enhancer of Zeste Homolog 2) appears common in both localized and metastatic prostate cancer, and associated with poor prognosis (27, 28). In early prostate carcinogenesis, during the transition from benign prostate epithelium to inflammatory lesions, DNA hypermethylation is observed in the promoter regions of key tumor suppresser genes such as GSTP1, RASSF1A, and APC (29, 30). In TRAMP mice and TRAMP-C1 cell line, hypermethylation of Nrf2's CpGs occurred (31), and global alteration of epigenomic DNA methylation was found with profiling using MeDIP-seq in TRAMP prostate tumor (32). In a PTEN deletion mouse prostate carcinogenesis model, PTEN deletion drives aberrations of DNA methylome and transcriptome in different stages of prostate carcinogenesis (33). Prostate cancer exhibited global alterations of histone modifications, including histone acetylation (H3K9, H3K18, and H4K12) and methylation (H3K4me2) (34). Gerhauser et al. (35) investigated the molecular evolution of 292 early-onset prostate cancer patients using Illumina 450K methylation array identified some unique DNA methylation patterns of 500 most discriminatory CpG sites between the different cell types (basal, stromal, normal luminal and tumor luminal) and found that the "purity-adjusted epigenetic prostate cancer index" (PEPCI) associated with increased Gleason score (36). By integrating the DNA methylation and RNA expression data from tumors diagnosed with early-onset, they were able to identify four robust subgroups which could stratify the patients into high- and low-risk groups(35).

Epigenetics plays an increasing role in the pathophysiology of colorectal cancer (CRC) (37). In an AOM-DSS induced colitis-accelerated colon cancer mouse model, differential CpG methylome and transcriptome occurs as compared to control mice (38). CpG island methylator phenotype (CIMP), has been identified as one of the molecular features of CRC (39, 40). Based on CIMP profiles, primary CRC may be clustered into three distinct but relatively homogeneous subclasses: CIMP1, characterized by intense methylation of multiple genes including MSI and BRAF mutations; CIMP2, increased methylation with age-related genes, and mutations in KRAS; and CIMP negative, characterized by rare methylation with p53 mutation (39). CIMP1 and CIMP2 phenotypes are more often expressed in the proximal colon; CIMP1 has a good prognosis, whereas CIMP2 has a poor prognosis (41). In general, CIMP appears to be associated with significantly worse prognosis in CRC patients (42).

Overview of epigenetic mechanisms and their role in cancer prevention

Epigenetic mechanism appears to provide a robust means for organisms to respond to environmental cues through changes in gene expression (43), working in concert with other more rapid responders including channels/receptors mediated signaling. These post-translational modifications act in a coordinated fashion leading to chromatin conformational alterations that, in turn, regulate the genetic information accessed by transcription factors (44). The schematic representation of epigenetic pathways in stepwise carcinogenesis process and cancer prevention by isothiocyanates (ITCs) impinging upon and integrating with the different cellular processes are summarized in Figure 1. Epigenetic machinery would affect all the stages during carcinogenesis. Among the three epigenetic mechanisms, DNA CpG methylation is probably the most studied as reviewed by Feinberg (6). Specifically, DNA methylation entails the conjugation of methyl groups in cytosine (C) residues usually occur in CpG dinucleotide sequences, dispersed across the genome, leading to the formation of 5-methylcytosine (5mC) (45). DNA methylation is catalyzed by a class of enzymes known as DNA methyltransferases (DNMTs) the most predominate which include DNMT1, DNMT3A and DNMT3B. Unlike methylation, histone acetylation causes gene activation whereas histone methylation can result in either activation or repression of genes depending on the site of modifications (46). Histone deacetylases (HDACs), along with histone acetyltransferases (HAT), provide a significant mechanism of gene regulation involving removal and addition, of acetyl groups from an ϵ -N-acetyl lysine amino acid on histone proteins. The third epigenetic control would be regulated through non-coding-RNA-based mechanisms including micro-RNA. In particular, micro-RNA transcription can be regulated by both histone modification and DNA methylation, and micro-RNA themselves can, in turn, regulate key enzymes that enforce epigenetic remodeling (47).

Several chemotherapeutic drugs targeting epigenetic signaling have been approved by the FDA (14), and a growing list of dietary phytochemicals found naturally in food and plants have been studied targeting epigenetic mechanism during the carcinogenesis process for cancer prevention including modifications of histones and DNA methylations (48, 49). For instance, curcumin, a powerful anti-inflammatory/antioxidant agent, regulating multiple signaling pathways, is also found to modulate epigenetics/epigenomics pathways such as histones modifications, and DNA methylation (50, 51). Many natural products have been

reported to inhibit the expression of HDACs, including curcumin (52, 53), ursolic acid (54), and SFN (55, 56). Triterpenoids such as cucurbitacin, ginsenoside Rh2, ginsenoside compound K and ursolic acid, have been shown to induce global hypomethylation, enhances hypermethylation/hypomethylation of the promoters of oncogenes and tumor suppressor genes, and modifying miRNA by targeting DNMT1 (48). SFN could regulates DNA demethylation by down-regulating the expression of DNMT1 and DNMT3B, thus inducing the demethylation of the cyclin D2 gene promoter and its expression in cancer cells (57). Question remains as to how all these chemopreventive phytochemicals modulate the epigenetic machinery. Since many of these compounds are redox active, therefore, we would like to postulate that redox signaling triggered by these compounds could play a role in modulating the epigenetic machinery, but further study would be needed.

Role of redox signaling in the modulation of epigenetic machinery

Cellular oxidative stress occurs when the cellular antioxidant/reductive capacity is overwhelmed by the oxidative challenges (58, 59), and could play a vital role in epigenetic reprogramming (60). Many epigenetic marks, including histone methylation, histone acetylation, ADP-ribosylation and DNA methylation, may be directly associated with central metabolism through redox intermediates (60). One mechanism by which reactive oxygen species (ROS) can affect DNA methylation is through its action on DNMTs activity or expression (61). In an oxidizing redox chromatin microenvironment and in the absence of S-adenosyl methionine (SAM), DNMT3a and DNMT3b can catalyze the direct conversion of 5mC or 5-hydroxymethylcytosine (5hmC) to an unmodified C, although the exact mechanisms are still unclear (62). Ye et al. have proposed an iron–redox–methylation hypothesis (63) in which excess amount of irons perturbs DNA methylation by at least two possible mechanisms: first, oxidative stress depletes GSH and drives SAM/SAH cycle to produce more SAH, which inhibits DNMT activity; and second, iron directly inhibits DNMT activity. The methylation process can be reversed by DNA demethylases such as ten-eleven translocation enzymes (TETs) (64). ROS are also thought to regulate DNA methylation by targeting the expression and/or activity of the TETs. Knockdown of TET1 significantly increase hydrogen peroxide-induced apoptosis of cerebellar granule cells, and cerebellar granule cells from Tet1 knockout mice are more sensitive to oxidative stress (65). In addition, mutagenic lesion of ROS-induced DNA damage of O⁶-methylguanine can inhibit binding of DNMT leading to hypomethylation with inhibition of methylation of adjacent cytosine nucleotides (66). Incorporation of 8-OHdG and the oxidation by-product of 5-mC in the Methyl-CpG binding protein (MBP) recognition sequence resulted in significant inhibition of the binding affinity of MBP (67).

In addition to epigenetic regulation of DNA methylation, redox also participates in histone acetylation and histone methylation. The histone deacetylation is mediated HDACs. A study has shown that the redox-regulating protein Thioredoxin 1 (Trx1) regulates the nucleocytoplasmic shuttling of class II HDACs through a redox-dependent mechanism (68). By forming a multiprotein complex with DnaJb5, a heat shock protein 40, and TBP-2, a Trx1-binding protein, Trx1 reduces HDAC4, a class II HDAC, at Cys-667 and Cys-669, which are easily oxidized to form a disulfide bond in response to hypertrophic stimuli(69). Hu, et al. showed that the catalytic activity of HDAC5 suppresses mitochondrial ROS

generation and subsequent induction of NRF2-dependent antioxidant gene expression in cardiomyocytes (70). Collectively, there is strong evidence that ROS are capable to modulate chromatin accessibility by affecting histone acetylation state in particular via multiple modifications of HDAC expression and activity (71, 72). Histone acetylation is reversible and is regulated by a group of histone acetyltransferases (HATs) (73). Histone methylation is maintained by two classes of enzymes: histone methyltransferases (HMTs) and histone demethylases (HDMs) (74). HMTs and HDMs can also be subjected to redox regulation such that post-translational modifications of histone proteins by oxidants and environmental stresses can trigger gene transcription involved in chronic inflammatory events (75). Since many dietary chemopreventive phytochemicals possess redox (76, 77) and epigenetic/epigenomic properties (78), question remains the direct linkage of phytochemical's redox signaling and epigenetics/epigenomics.

Isothiocyanates and cancer prevention

Isothiocyanates (ITCs) are a family of compounds derived almost exclusively from plants, although marine sponges and fungi also have been reported to produce a few ITCs (79, 80). ITCs are synthesized and stored as glucosinolates (β -thioglucoside N-hydroxysulfates) as secondary metabolites in plants including the cruciferous. Glucosinolates are relatively stable in plant cell. However, when the plant tissue containing glucosinolates is damaged, as in the case of preparation (cutting, chopping, mixing) or chewing food, a β -thioglucosidase called myrosinase is released and hydrolyzed the glucosinolates to various metabolites, including the ITCs (81). When cruciferous are cooked before consumption, myrosinase is inactivated and glucosinolates transit to the colon where they are hydrolyzed by the intestinal microbiota to ITCs (81). By far the most studied ITC is sulforaphane (SFN), derived from broccoli (80), although other ITCs, including phenylethyl ITC (PEITC), benzyl ITC (BITC) and allyl ITC (AITC), also possess chemopreventive properties. Multiple molecular mechanisms are involved in the chemopreventive effect of ITCs. Below, we will focus on epigenetic mechanisms in *in vitro* and *in vivo* studies in skin, breast, lung, colon and prostate cancer, as well as some clinical chemoprevention studies with ITCs.

Skin cancer

The ITCs show chemopreventive effects against UV- and chemically-induced skin carcinogenesis (82) and the involvement of the epigenetics in epidermal carcinogenesis has been studied in melanoma (83). The activity of SFN on epigenetic regulation of skin cancer has been reported on PcG proteins' (e.g. Bmi1 and EZH2) function by means of blocking tumor progression through their inhibition thus decreasing H3K27me3 level. (84). In squamous cell carcinoma (SCC), SFN treatment suppressed cancer progression and metastasis *in vivo* through reduction in arginine methylation at H3 (85). As discussed earlier, UVB would drive perturbation of CpG methylome at initiation, promotion and progression stages of skin carcinogenesis, but importantly, SFN would block carcinogenesis more effectively at early stages than late stages and would attenuate some of the perturbations of UVB-induced CpG methylome (86). In cell culture model, SFN was also found to demethylate CpGs of Nuclear factor erythroid 2-related factor 2 (NRF2) promoter, resulting in re-activation of NRF2-dependent expression of detoxification enzymes heme oxygenase 1

(HO-1), NAD(P)H quinone oxidoreductase 1 (NQO-1), and UDP-glucuronosyltransferase 1A1 (UGT1A1) potentially leading to inhibition of TPA-induced transformation in epidermal JB6 cells (87). Such induced NRF2 expression was attributed to promoter hypomethylation through down-regulation of DNMTs and inhibition of overall HDAC enzyme activity (87). Professor Paul Talalay who first isolated and identified SFN (88), and he also advanced the concept of redox/electrophile-mediated induction of phase 2 detoxifying genes (89). Oxidants and electrophiles including SFN could modify cysteine's sulfhydryl groups of Kelch-like ECH-associated protein-1 (KEAP1), prevent proteasomal degradation, allow newly synthesized NRF2 to enter the nucleus, and enhance NRF2-target gene transcription (reviewed in (90–92)). Interestingly, however, electrophile such as SFN and oxidant such as H₂O₂ may also regulate NRF2 via redox sensitive cysteine on the Nuclear Export Signal (NES) (93) as well as post-transcriptional/translation control of NRF2's Internal Ribosomal Entry Sites (IRESs) (94). Question remains, how these different redox signaling of NRF2 would operate under different redox environments such as different redox agents, acute versus chronic redox agent's stimulating conditions and pathological conditions.

Lung cancer

Several studies show an inverse relationship between consumption of dietary ITCs, and lung cancer risk (95). *In vitro* cell culture study shows the involvement of SFN and miR-616–5p in EMT and NSCLC metastasis (96). miR-616–5p directly targets GSK3 β and decreased its expression, whereas SFN decreased miR-616–5p by histone modification, and followed by inactivation of the GSK3 β / β -catenin signaling pathway and inhibition of EMT in NSCLC cells (96). SFN inhibits HDAC activity and increases acetylated histones H3 and H4 in A549 and H1299 lung cancer cells (97). In lung cancer stem cells (CSCs) miR-19a and miR-19b expression are up-regulated and SFN suppresses miR-19 and Wnt/ β -catenin pathway resulting in inhibition of lung CSCs (98). In human lung cancer A549 cells, SFN epigenetically demethylates the CpG sites of the miR-9–3 promoter and reactivates miR-9–3 expression (99). Similarly, SFN was found to up-regulate miR-214 and mediate downregulation of c-MYC in non-small cell lung cancer, potentially results in inhibition of cancer stem-like cell properties and cisplatin resistance (100).

Colon cancer

In colon cancer model, SFN suppressed tumor development in Apc(min) mice, increased acetylated histones in the polyps, and inhibited HDAC in APC(min) mice (101). SFN treatment in human colon adenocarcinoma Caco-2 cells showed increased activation of NRF2 by demethylation of the NRF2 promoter region and reducing expression of DNMT1. (102). In polyposis in rat colon (Pirc) model, single oral administration of SFN and structurally related long-chain ITCs decreased HDAC3 expression and increased pH2AX levels in adenomatous colon polyps (103). HCT116 and SW480 colon cancer cells study showed that SFN or its analogs altered HAT/HDAC activities and histone acetylation status, lowered the expression of HDAC3, P300/CBP associated factor (PCAF) and lysine acetyltransferase 2A (KAT2A/GCN5), and attenuated homologous recombination (HR)/non-homologous end joining (NHEJ) repair activities (103). A 0.12% PEITC-enriched mouse-diet reduced mucosal and submucosal inflammation possibly via modulating regulating NF κ B proteins and NF κ B mRNA expression was inversely correlated with tri-methylation

of lysine 27 on histone 3 near its promoter region in a time-dependent manner (104). Interestingly, colon cancer cells treated with low-dose PEITC for >1 month exhibited stable alterations in expression profile of epigenetic writers/erasers and chromatin-binding of HDACs and Polycomb-group (PcG) proteins (105). Sustained PEITC exposure not only blocked HDAC protein binding to euchromatin but was also associated with hypomethylation of PcG target genes that are typically hypermethylated in cancer (105). In human colon cancer cells ITCs inhibit HDAC enzyme activity and increase HDAC protein turnover and ITCs enhanced the acetylation and subsequent degradation of critical repair proteins, such as CtIP (106).

Breast cancer

Studies show that SFN modulates breast cancer risk at multiple stages of carcinogenesis through different biologic mechanisms including epigenetic modulations (107). SFN inhibited in dose- and time-dependent of hTERT in both MCF-7 and MDA-MB-231 human breast cancer cells. DNMT1 and DNMT3a, were decreased in SFN-treated breast cancer cells suggesting that SFN may repress hTERT by impacting epigenetic pathways (108). In combination studies, SFN and EGCG activated ER α MDA-MB-231 cells which was associated with significant reduction of DNMTs expression and activity, as well as of HDAC activity (109). Synergistic effect on MDA-MB-231 xenograft tumor growth, with combination of SFN and LSD1 inhibitor (110). In transplacental breast cancer chemoprevention study, SFN containing broccoli sprout diet upregulates tumor suppressor genes p53 and p16INK4a and downregulates tumor-promoting genes TERT and c-Myc potentially as a result of inhibition of HDAC1 expression (111). SFN in combination with genistein downregulates HDAC2, HDAC3 and KLF4 protein expression and reduced tumor volumes/sizes (112).

Prostate cancer

In prostate cancer, SFN reactivated and induced its downstream antioxidant stress pathway in TRAMP-C1 cells through epigenetic modification of NRF2 promoter, and could play a role in chemoprevention (113). The expression of long noncoding RNAs (lncRNAs) in prostate cancer cells was altered and SFN restored the altered expression of lncRNAs (114). Increased SFN consumption in mice with prostate cancer cell xenografts showed reduced HDAC enzyme activity in prostates, and peripheral blood mononuclear cells (115). SFN inhibited the expression and activity of human telomerase reverse transcriptase (hTERT). SFN treatment selectively decreased HDAC activity, and Class I and II HDAC protein's expression increased acetylated histone H3 at the promoter for P21, induced p21 expression and increased tubulin acetylation in prostate cancer cells (116).

Cancer chemoprevention by isothiocyanates (ITCs) via non-epigenetic signaling pathways

Numerous studies have shown that many non-epigenetic mechanisms by which ITCs exert their biological anti-carcinogenic activities have been being well investigated including induction of carcinogen detoxication (117), enhanced DNA damage repair (118, 119), anti-inflammatory pathway through NF- κ B regulation (120), elimination of cancer stem cells

(121) and other cells (122), inhibition of cell cycle progression (123), induction of caspase/apoptosis (124), induction of autophagy (125), and MAPK signaling pathway (126), among others. As discussed above, the most studied and most relevant signaling pathway in the context of cancer prevention elicited by ITCs would be the NRF2 mediated signaling pathway. As reviewed by many scientists, and discussed above, ITCs react with sulfhydryl groups of C residues of Keap1, releasing NRF2 from Keap1 binding and then NRF2 translocating to the nucleus, partnering with sMafs and binding to the ARE found in many antioxidant/detoxifying enzymes including glutathione S-transferases (GSTs), thioredoxin, NQO-1, HO-1, among others (127–131).

Many studies have also shown that the ITCs are potent anti-inflammatory agents. SFN significantly reduced the nuclear translocation of the pro-inflammatory transcription factor nuclear factor (NF)- κ B in pancreatic acinar cells, downregulating the expression of NF- κ B target genes that code for pro-inflammatory mediators, such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1 β), IL-6 and inflammatory enzymes cyclooxygenase-2 (COX-2), prostaglandin E (PGE) synthase, and inducible nitric oxide synthase (iNOS) (120, 132). ITCs can also modulate the activity of phase I metabolizing enzymes (133–135), such as downregulating CYP3A2 mRNA expression, as well as the activity of benzyloxyquinoline debenzylase, a marker of CYP3As and upregulating CYP1A1 and CYP1A2 mRNA expression and the activity of ethoxyresorufin-*O*-deethylase (EROD), a marker of CYP1A1/2 activities (136). Some procarcinogens require activation by phase I enzymes in order to become active carcinogens capable of binding DNA and forming cancer-causing DNA adducts (137). Inhibition of specific CYP enzymes involved in carcinogen activation has been found to prevent the development of cancer in animal models (138). In addition, ITCs have been shown to modulate the expression of cell cycle regulators, cyclins and cyclin-dependent kinases (CDK), and trigger apoptosis in cancer cell lines (139). For example, in a mouse model of CRC, PEITC administration reduced both the number and size of polyps and these alternations were associated with activation of the CDK inhibitor, p21, inhibition of various cyclins (A, D1, and E), and induction of apoptosis (140). ITCs also have been shown to suppress the formation of capillary-like structures from human umbilical endothelial cells and likely inhibit the expression of hypoxia inducible factors (HIFs) that control angiogenesis in endothelial cells and malignant cell lines inhibition of angiogenesis (141). As discussed above for BITC (142), SFN has been shown to induce autophagy in both *in vitro* cell culture models and *in vivo* models associated with decreased cell proliferation, alterations in protein levels of autophagy regulators Atg5 and phospho-mTOR (125, 143). Some other anticancer pathways such as inhibition of cell migration and invasion (144–146), have also been reported. Question remains how these non-epigenetic signaling pathways would be linked and integrated with epigenetic mechanisms elicited by ITCs in the context of cancer prevention necessitates further study.

Pharmacokinetics and Pharmacodynamics of dietary ITCs

Pharmacokinetics (PK) and pharmacodynamics (PD), a quantitative science, integrate and evaluate the dose/concentration-response relationships in the body following the administration of drugs (147, 148). Despite many reports studying the PK/PD of ITCs, there are not many showing the “direct quantitative relationship” between the dose/concentration

of ITCs and the biological/pharmacological response(s) in *in vivo* animal models or in humans. In absorption, after oral administration of SFN in the rats the peak concentration (C_{max}) were reported to be attained in 1 h, indicating rapid absorption (149). However, increasing the dose of SFN from 0.5–5 mg/kg, the oral absorption rate constant (k_{ab}) decreased and C_{max} did not increase proportionally to the dose, suggesting non-linear absorption kinetics (149). In human, SFN was absorbed rapidly and achieved C_{max} in 1 h after the consumption of broccoli sprout products (150, 151). Comparison of the area under the blood/plasma concentration time curve (AUC) AUC_{0-24} values between the intravenous (IV) and oral treatment groups, at low dose (2.8 $\mu\text{mol/kg}$) shows an absolute bioavailability of 82%, which decreased to 20% at the higher dose (28 $\mu\text{mol/kg}$) (149).

In drug distribution, a rapid decrease was observed in the plasma concentration of SFN after both oral and IV dosing indicating rapid and extensive cellular uptake into various tissues (149). This is consistent with studies showing ITCs achieve very high intracellular concentrations as a result of their interaction with glutathione (GSH) (152–154). Such extensive intracellular localization explains the very large apparent volume of distribution (V_d) of 102 L/kg after IV injection in rat. Additionally, the V_d of SFN decreased dramatically from 102 to 42 L/kg with increasing doses from 0.5 to 5.0 mg/kg (149), showing non-linear V_d . In another study of SFN PK, the volume of distribution at steady state (V_{ss}) was around 3.7 L or 14.8 L/Kg after an IV dose of 25 mg/kg of SFN (155) and this result is consistent with Hanlon et al.'s findings (149).

Freeze-dried aqueous extract of broccoli sprouts inhibited bladder cancer development induced by N-butyl-N-(4-hydroxybutyl) nitrosamine significantly and dose-dependently, with over 70% of the ITCs present in the extract were excreted in the urine as ITC equivalents (ITCs + dithiocarbamates) within 12 h after a single oral dose, indicating substantial urinary excretion (156). Urinary concentrations of ITC equivalents were 2 to 3 orders of magnitude higher than those in plasma (156). In a cross-over human study of glucoraphanin-rich (GRR) versus SFN-rich (SFR), urinary excretion of SFN and its metabolites (in approximately 12-hour collections after dosing), was substantially greater with the SFN-rich (mean = 70%) than with GRR rich (mean = 5%) beverages (157). These studies again suggest that substantial renal excretion of ITC or its metabolites occur.

In terms of PD, several studies have reported the increased levels of Nrf2-targeted enzymes GSTs and NQO1 in plasma (158) and saliva (159) in humans volunteers after consuming cruciferous vegetables. Similarly administration of SFN-rich preparations to healthy subjects led to increased mRNA or protein levels of NQO1 and GSTs in skin punch biopsies, blood cells, and buccal scrapings (160–162). As a proof of concept study, after IV administration of 25 mg/kg of SFN, moderate increase in mRNA (2–5 folds) of HO-1, NRF2, and NQO1, while significant increase (> 5 folds) for GSTT1, GPx1, and Maf in rat lymphocytes (155). These PK/PD effects of SFN could be modeled with indirect response (IDR) model directly linking plasma SFN concentrations with NRF2-mediated gene expression response in lymphocytes (155). Future studies involving integrating acute PK/PD and long-term cancer preventive effects of ITCs in human would be needed.

Ongoing clinical studies/trials of isothiocyanates and chemoprevention

Pre-clinical carcinogenesis studies of dietary ITCs have demonstrated extraordinary chemopreventive efficacy. Several clinical trials have illustrated that ITCs, administered orally as glucosinolate precursors or directly in its bioactive compound such as SFN, PEITC have preventive effects against cancers (163) (164). Specifically, SFN and PEITC have been studied in some clinical studies and briefly summarized in Table 1 (165–171). More ongoing ITCs clinical trials are listed in clinicaltrials.gov. In one particular clinical study involving male patient with recurrent prostate cancer, none of the patients experienced PSA doubling, a marker for disease severity, after serving SFN daily for 20 weeks (172). HDAC expression and activity inhibition has been used as a biomarker in some trials (173, 174) and one clinical study with healthy subjects showed that HDAC activity was significantly inhibited in PBMCs as early as 3 hours after consumption of 68 g of broccoli sprouts (115). Study using SFN in breast cancer patients showed an average decrease of 80.39 pmol/min/mg protein in blood HDAC activity from pre- to post-intervention (169). A clinical study of PEITC as an inhibitor of metabolic activation of a tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) for cancer prevention, the NNK metabolic activation ratio was significant reduced by 7.7% (171). ITCs have shown promising efficacy in various clinical cancer chemoprevention trials, however, future clinical trials involving epigenetic endpoints are warranted.

Conclusion and future prospective

ITCs found abundantly in the human diet and are derived from cruciferous vegetables, are highly promising cancer preventive agents. Multiple and multi-targeted mechanisms, including epigenetic reprogramming, could be involved in the preventive and therapeutic effects of ITCs in cancer. Gene silencing through hypermethylation of CpG of tumor onco/proto-oncogenes, gene activation through hypomethylation of CpG of tumor suppressor genes, and other modifications including phosphorylation, acetylation, ubiquitination are potential cellular targets of epigenetic regulation by ITCs. Genome-wide DNA methylation analysis might help to better understand the targeted tissue specificity and global events in the regulation by ITCs. Extensive research on the extrapolation of preclinical to clinical dosages of ITCs for prevention studies needs to be further refined. Understanding of the PK/PD of parent/metabolites of the ITCs, its tissue levels and modulation of signaling mechanisms including epigenetic mechanisms is warranted in human studies.

Cancer is a complex chronic disease and cancer development is a multistep process (1, 2, 12, 175, 176). The ITCs as discussed above belongs to a very powerful class of biologically active food components, they are relatively non-toxic at low physiological and even higher pharmacological doses, with good oral bioavailability (149, 157), powerful anti-oxidative stress/anti-inflammatory (80) and possess epigenetic modifying properties as well as with great anti-cancer efficacy in many *in vitro* cell culture models and *in vivo* animal models. The exact molecular mechanism of how the ITCs as well as many other dietary chemopreventive phytochemicals modifying the epigenetic machinery is currently unclear. However it is highly likely through redox biological process playing an important role (60) analogous to the kinases/caspases activation as we have discussed back in the 1990s (177–

179). Additionally, it would be tempting to speculate that the ITCs particularly SFN and PEITC, although differ in some aspects of signaling pathways (at same micromolar concentrations, SFN is a more potent NRF2 activator than PEITC, and SFN activates ERK more strongly compared to JNK, whereas PEITC would be reversed (126, 180, 181), they would be considered as “general” cancer preventive agents not unlike the chemotherapeutic agents such as platinum-based or the taxane-based drugs. Analogous to chemotherapeutic agents, for maximal efficacy, the ITCs would need to be used in combination with other cancer preventive agents including other less toxic dietary phytochemicals, natural products or drugs such as NSAIDs, SERMs, aromatase inhibitors, and 5- α -reductase inhibitors (182) targeting different signaling pathways. Together with other considerations and initiatives such as immunoprevention (183, 184) (185), the long-term control and prevention of progression of tumors to advanced and metastatic states may soon to be realized.

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Abbreviations:

ITCs	Isothiocyanates
SFN	Sulforaphane
GS	glucosinolates
EMT	Epithelial-Mesenchymal Transition
NSCLC	Non-Small Cell Lung Carcinoma
hTERT	human Telomerase Reverse Transcriptase
OSCC	Oral Squamous Cell Carcinoma
NQO1	NAD(P)H: quinone oxidoreductase
HO-1	hemeoxygenase 1
NRF2	Nuclear Factor Erythroid-2-related Factor-2
NF-κB	Nuclear Factor Kappa B
DNMT-1	DNA methyltransferase 1
EGFR	Epidermal Growth Factor Receptor

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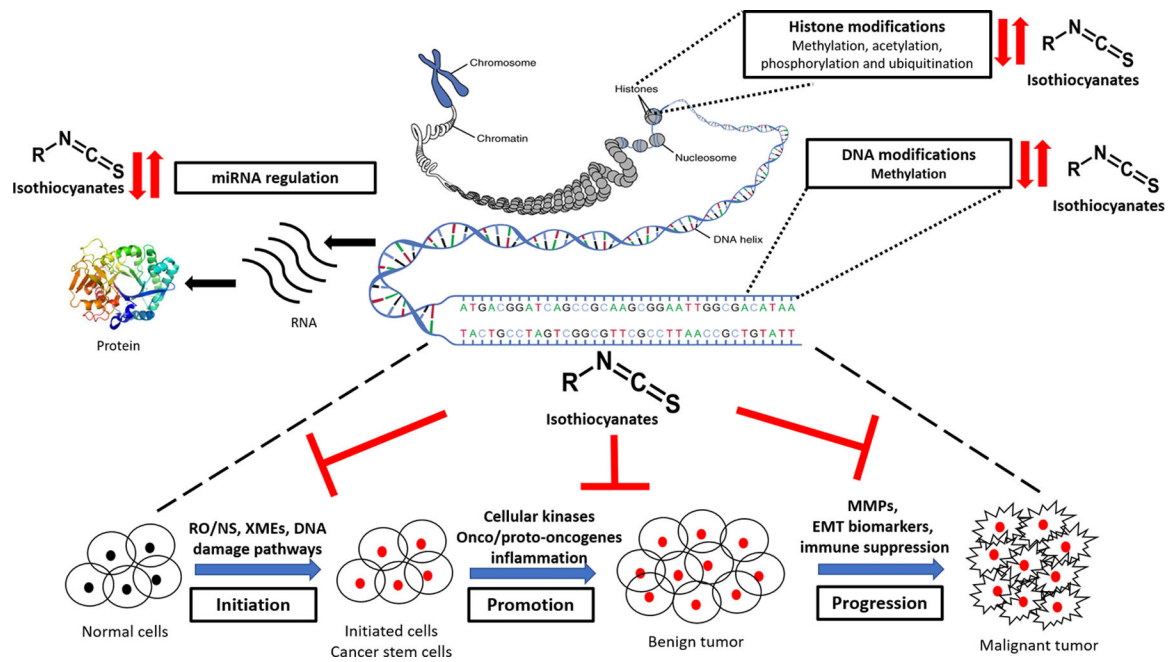


Figure 1.

Schematic representation of epigenetic pathways in stepwise carcinogenesis process and chemopreventive effects of isothiocyanates (ITCs) impinging upon and integrating with the cellular processes during cancer development. Major epigenetic mechanism involved (A) Histone modification, (B) DNA modifications, and (C) miRNA regulation. The role of ITCs in modifying/reversing of these epigenetic mechanisms and signaling pathways including reactive oxygen/nitrogen species (RO/NS), xenobiotic metabolizing enzymes (XMEs), DNA damage/repair, cellular kinases, onco/proto-oncogenes, inflammatory, matrix metalloproteinases (MMPs), epithelial-mesenchymal transition (EMT) biomarkers, and immune suppression, among others, in the process of carcinogenesis viz. initiation, promotion and progression. (↑ activation, ↓ inhibition, ⊥ blocking)

Table 1:

Summary of completed and ongoing clinical trials using SFN and PEITC

Agent	Cancer type	Experiment detail	Outcome/Ongoing targets
SFN/Broccoli Sprout Extract	Lung	291 healthy subjects; broccoli sprout beverages; GR (600 µmol) and SFN (40 µmol); 84 days	Increases urinary excretion of benzene (61%) and acrolein (23%) (165)
		50 healthy subjects; SFN-rich broccoli sprout beverage; 7 days	20–50% ↑ in excretion levels of glutathione conjugates of acrolein, crotonaldehyde and benzene after SFN treatment (166)
		30 healthy subjects, young smokers; broccoli diet; 250 g/ day; 10 days	DNA repair activity ↑ in PBMC (167)
		72 former smokers with lung cancer; SFN; 120 µmol/time, 2 times/day; 12 months	Targets: lung cancer chemoprevention with SFN in former smokers
	Prostate	90 men with prostate cancer recurrence; 60 mg of SFN daily; 6 months	log PSA slope ↓ significantly (168)
		98 men with prostate biopsy; 200 µmol of SFN, 2 capsules daily; 4 weeks	Targets: HDAC inhibition effects by SFN in human prostate cancer tissue/cells
		40 prostate cancer patients; 64 mg SFN daily, 2 times daily; 4 weeks	Epigenetic biomarkers measurement after SFN treatment
	Breast	54 breast biopsy subjects; 224 mg GR; Broccoli seed extract;	Ki67 ↓, HDAC3 ↓ in benign tissue, HDAC ↓ in PBMC (169)
34 DCIS breast cancer subjects; 100 µmol of SFN for 14 days		Change in Mean Proliferative Rate Measured by Ki67%	
PEITC/Watercress	Lung	11 healthy smokers; watercress; 56.8 g / meal for 3 meals/day; 3 days	↑ Urinary NNAL plus NNAL-Gluc (33.5%) (170)
		82 healthy smokers; PEITC 10 mg 4 times/day for 5 weeks	↓ NNK metabolic activation ratio (7.7%) (171)

SFN, sulforaphane; PEITC, phenethyl isothiocyanate; GR, glucoraphanin; PBMC, Peripheral blood mononuclear cells; PSA, prostate-specific antigen; HDAC, Histone Deacetylase; NNAL-glu, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butyl /3-D-glucopyranosiduronic acid; NNK, Nicotine-derived nitrosamine ketone; DCIS, Ductal carcinoma in situ; ↑, increase/activation; ↓, decrease/inhibition.