Temporal Efficacy of a Sulforaphane-Based Broccoli Sprout Diet in Prevention of Breast Cancer through Modulation of Epigenetic Mechanisms

Yuanyuan Li1,2,3, Phillip Buckhaults4, Shizhao Li5, and Trygve Tollefsbol2,3,5,6

Abstract
Breast cancer is the most common cancer and the second leading cause of cancer-related death among women. An important risk factor for breast cancer is individual genetic background, which is initially generated early in human life, for example, during the processes of embryogenesis and fetal development in utero. Bioactive dietary components such as sulforaphane (SFN), an isothiocyanate from cruciferous vegetables including broccoli sprouts (BSp), cabbage, and kale, has been shown to reduce the risk of developing many common cancers through regulation of epigenetic mechanisms. Our study indicates a prenatal/maternal BSp dietary treatment exhibited maximal preventive effects in inhibiting breast cancer development compared with postnatal early-life and adult BSp treatments in two transgenic mouse models that can develop breast cancer. Postnatal early-life BSp treatment starting prior to puberty onset showed protective effects in prevention of breast cancer but was not as effective as the prenatal/maternal BSp treatment. However, adulthood-administered BSp diet did not reduce mammary tumorigenesis. Our results suggest that the prenatal/maternal BSp bioactive natural plant product may impact early embryonic development by regulating global differential gene expression through affecting epigenetic profiles resulting in differential susceptibility to breast cancer later in life. These results suggest that a temporal exposure to epigenetic-modulating dietary components such as cruciferous vegetables could be a key factor for maximizing chemopreventive effects on human breast cancer. This study may lead to translational breast cancer chemopreventive potential by appropriate administration of key dietary components leading to early breast cancer prevention in humans. Cancer Prev Res; 11(8); 451–64. ©2018 AACR.

Introduction
The etiology of most human diseases involves complicated interactions of multiple environmental factors with individual genetic and epigenetic background, which is initially generated early in human life, for example, during the processes of embryogenesis and fetal development in utero (1–4). Breast cancer is the most common cancer in women in the United States and has been closely linked to inherited tendency as well as certain environmental exposures (5, 6). Individual susceptibility to breast cancer can be influenced by exposure to certain environmental factors such as the diet during the lifetime (7).

Early embryogenesis includes a series of programming processes involving extremely accurate time-controlled gene activation/silencing expression leading to cellular differentiation and organisal development in different developmental stages. Epigenetic mechanisms including histone modifications and DNA methylation play major roles during early development in controlling sophisticated gene expression patterns via changing chromatin structure and the accessibility to transcriptional regulation (4). This includes DNA methylation reprogramming during early embryogenesis through widespread demethylation and subsequent de novo methylation processes to establish a unique gene-specific methylation pattern in the progeny. It has become increasingly apparent that dysregulation of epigenetic mechanisms during early embryogenesis is...
closely related to multiple developmental or congenital diseases as well as phenotypic impacts in later life such as different susceptibility to tumorigenesis (2). Certain environmental exposures during this critical period may affect early embryonic development and subsequent phenotypes of the progeny via, at least in part, epigenetic mechanisms (8). Thus, this vulnerability to environmental exposure during embryogenesis may provide an excellent opportunity to reprogram epigenetic profiles leading to potential beneficial outcomes such as disease prevention in the offspring.

Maternal exposure to certain diets with properties in influencing epigenetic processes could bridge the connection from mother to fetus via transplacental effects (7-9). Maternal diets may influence the epigenetic reprogramming processes during early embryogenesis, which may consequently influence gene expression patterns and eventually affect phenotypic outcome in the offspring such as differences in disease susceptibility. For example, a soy-rich maternal diet may modulate the methylome or acetylome to protect against the risk of developing obesity in the offspring, although the mechanisms responsible for this process are not yet fully understood (8).

The bioactive dietary component, sulforaphane (SFN), an isothiocyanate derived from glucoraphanin and enriched in cruciferous vegetables such as broccoli sprouts (BSp), is a strong epigenetic modulator and robust chemopreventive agent both in vitro and in vivo against various human diseases including breast cancer (10-13). Mechanisms involved in SFN and BSp-induced chemopreventive effects include induction of cell-cycle arrest, apoptosis, and activation of phase I CYP enzymes and phase II detoxification enzymes leading to restored mitochondrial function and reduced lipid peroxidation (14-16). Interest in SFN has been recently growing due to its potency for influencing epigenetic processes through targeting key epigenetic modulators such as histone deacetylases (HDAC) and DNA methyltransferases (DNMT), which may lead to local or global alterations of epigenetic hallmarks resulting in subsequent gene transcription and expression level changes (12, 13, 17, 18). Our previous studies have shown that SFN can induce repression of human telomerase reverse transcriptase (hTERT) and reactivate estrogen receptor (ER) in ER-negative breast cancer cells through epigenetic mechanisms (12, 13). Orally fed BSp diet can also cause global increase in histone acetylation and HDAC inhibition in breast xenograft mice (13, 18). These results indicate that beneficial botanicals such as cruciferous vegetables can lead to lower susceptibility to breast cancer by epigenetically impacting key tumor-related gene expressions.

Although research on SFN and its enriched BSp diet is very promising for chemoprevention of many cancers, determining their effectiveness for cancer chemoprotection during in utero and lactational exposure is still a challenging task. Interestingly, epidemiologic studies in the Polish who consume cabbage at three times more than other nationalities show a lower incidence of breast cancer especially when early consumption occurs during adolescence as compared with adult consumption (19). This result indicates that early-life consumption of SFN from cruciferous vegetables may be more effective than later-life consumption in preventing breast cancer. A study on carcinogen-induced tumorigenesis by Yu and colleagues revealed a greater protective effect of maternal dietary supplementation with indole-3-carbinol (I3C) from cruciferous vegetables on offspring survival, suggesting a chemopreventive effect of maternal cruciferous vegetable phytochemicals in vivo (20).

Based on these findings and others, we hypothesized that the BSp bioactive natural plant products may impact early development by affecting epigenetic profiles, resulting in different susceptibility to breast cancer later in life. In this study, we found that an incremental preventive effect on breast cancer in later life was correlated with an earlier temporal exposure to the BSp diet during the lifespan. In particular, prenatal/maternal exposure to dietary BSp led to maximal inhibition of breast cancer incidence in the offspring of transgenic mice as compared with postnatal early-life exposure, which was, in turn, more effective than adult exposure. Our results suggest that a lifespan temporal pattern for epigenetic-modifying dietary compounds may maximize effectiveness of their intervention outcome for breast cancer prevention in later life.

Materials and Methods

Animal diet preparation

A customized BSp diet (w/w, modified AIN-93G diet supplemented with 26% BSp; TestDiet) was prepared with AIN-93G diet base and adjusted for macronutrition content as used previously (13). TestDiet supplied all of the dietary ingredients except for the BSp, which was obtained from Natural Sprout Company. Dietary ingredients and nutrition profiles were provided in Supplementary Data (Supplementary Fig. S1). Diets were stored protected from light at –20° C throughout the feeding phase of the trial. Mice were given control and BSp diets as well as regular water ad libitum.

Animal models

We have used two transgenic mouse models including C3(1)-SV40 Tag (FVB-Tg[C3-1-Tag]cleg/legl]) (SV40) and FVB/N-Tg(MMTVneu)202Mu (Her2/neu) mice in our study. The female mice of these models can develop breast tumors caused by overexpressed transgenes at early ages (medium tumor latency is around 20 weeks for SV40 mice and 30 weeks for Her2/neu mice, respectively; ref. 21). The breeder mice at 4 weeks were obtained from The Jackson
Laboratory and bred from 8 weeks of age to obtain adequate colonies for this study. The Tag genotypes were identified at 21 days of life by analysis of tail DNA using standard PCR techniques according to the previous studies (22). All the mice were housed in the Animal Resource Facility of the University of Alabama at Birmingham (UAB; Birmingham, AL) and were maintained under the following conditions: 12-hour dark/light cycle, 24°C ± 2°C temperatures, and 50% ± 10% humidity. An online power calculator (http://powerandsamplesize.com/) was used to calculate the power/sample size by 2-proportion comparison based on our previous pilot studies.

Animal experimental designs

Prenatal/maternal BSp treatment (Pre-BSp). In the animal experimental design (Fig. 1), female transgenic mice (5–10 mice/group) were mated at 12 weeks of age and assumed to be pregnant when a vaginal plug was expelled. Prenatal/maternal BSp dietary administration began from conception and continued throughout pregnancy until the weaning period. Pups were weaned at postnatal 28 days of life (PD28), and the Tag genotype of the pups was determined by tail DNA analysis as described above. The mouse offspring were separated and maintained with control AIN-93G diet throughout their lifespan. Breast tumor observation and tissue extraction were performed in the offspring only. The control group diets were administered AIN-93G diet and continued throughout the study.

Postnatal BSp treatment. The transgenic mice at 4 weeks of age were randomly divided into two postnatal treatment groups (10–20 mice each). The experimental groups were designed as follows (Fig. 1): (i) early-life BSp group (Early-BSp): mice were fed with 26% BSp diet from 4 weeks of age and continued throughout the course of the study; (ii) adult BSp group (Adult-BSp): mice were fed BSp diet from 8 weeks of age and continued throughout the entire study.

Tumor observation and tissue collections

Both of these mouse models render the properties that can develop breast tumors at early ages (around 20 weeks in SV40 and 30 weeks in Her2/neu mice, respectively; ref. 21). Tumor latency, tumor size, and body weight were measured weekly. Tumor volumes were calculated using the formula: tumor volume (cm³) = (length × width²) × 0.523 (13, 18, 22). The experiment was terminated when the mean tumor diameter in the control mice exceeded 1.0 cm. At the end of the experiment, the primary breast

Figure 1.
Schematic representation of experimental design for BSp dietary treatments. The upper bar represents different life stages in the mother and female offspring. Transgenic mice were administered the BSp diet under different exposure time points: (i) Control: mice were fed ad libitum (AL) with the control AIN-93G diet; (ii) prenatal/maternal BSp treatment (Pre-BSp): The BSp diet was given to the mother from conception (12 weeks) until weaning (PD28); (iii) postnatal early-life BSp treatment (Early-BSp): The BSp diet from postnatal 4 weeks of age until termination of the experiment; (iv) postnatal adult BSp treatment (Adult-BSp): The BSp diet from postnatal 8 weeks of age until termination of the experiment. Mice were mated at 12 weeks of age following 3 weeks of gestation. Pups were weaned at 4 weeks of age (PD28). In the prenatal/maternal BSp group, weaned female offspring were maintained on the control diet throughout their lifespan until termination of the experiment and monitored for tumor growth weekly. For postnatal treatments, female mice were born to mothers administered the control AIN-93G diet and monitored for tumor growth weekly after weaning.
tumors were excised, weighed, and appropriately stored in liquid nitrogen for further analysis. Tissue specimens were snap frozen in liquid nitrogen for further studies such as RNA, DNA, and protein extraction. Animal procedures were reviewed and approved by UAB Institutional Animal Care and Use Committee (IACUC; Animal Project Numbers: 11010932 and 20671). All experiments and procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at UAB.

qRT-PCR
Snap-frozen mouse breast tumors (~20 mg) from each treatment group were thawed and RNA was extracted using standard protocol. Total RNA from tissues was extracted using the RNeasy Plus Universal Kit (Qiagen) according to the manufacturer’s instructions and reversely transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad) as performed previously (12, 13, 18, 22). Specific gene primers for p53, p16, TERT, c-Myc, HDAC1, Dnmt1, and GAPDH were synthesized and provided by Integrated DNA Technologies. Gene expressions were performed in triplicate and analyzed by real-time PCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a Bio-Rad CFX Connect Real-time System. Thermal cycling was initiated at 94°C for 15 seconds; 30 cycles of PCR (94°C, 15 seconds; 60°C, 30 seconds). GAPDH was used as an endogenous control, and vehicle control was used as a calibrator. The relative changes of gene expression were calculated using the following formula: fold change in gene expression, \[2^{-\Delta\Delta Ct} = 2^{-\Delta Ct(\text{treated samples}) - \Delta Ct(\text{untreated control samples})}\], where \(\Delta Ct = Ct(\text{test gene}) - Ct(\text{GAPDH})\) and \(Ct\) represents threshold cycle number.

Western blot analysis
Tissue proteins from mouse breast tumors were homogenized and extracted with T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific) according to the manufacturer’s protocols. Proteins were electrophoresed in Bio-Rad CEF Connect Real-time System. Thermal cycling was initiated at 94°C for 15 seconds, followed by 35 cycles of PCR (94°C, 15 seconds; 60°C, 30 seconds). GAPDH was used as an endogenous control, and vehicle control was used as a calibrator. The relative changes of gene expression were calculated using the following formula: fold change in gene expression, \[2^{-\Delta\Delta Ct} = 2^{-\Delta Ct(\text{treated samples}) - \Delta Ct(\text{untreated control samples})}\], where \(\Delta Ct = Ct(\text{test gene}) - Ct(\text{GAPDH})\) and \(Ct\) represents threshold cycle number.

Histologic analysis
Primary breast tumor tissues were dissected and fixed in 10% buffered-neutralized formalin for histologic analysis. Tumor slices (5 μm thick) were stained with H&E staining and evaluated by a licensed pathologist. From each H&E-stained section, microphotographs of three randomly selected fields were taken at a final magnification of ×200. Microscopic analysis of breast tissue sections was performed using an Olympus BX41 microscope fitted with a Q-color 5 Olympus camera.

HDAC activity assay
Nuclear proteins from mouse breast tumor tissues were extracted by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). The HDAC activity was evaluated by EpiQuik HDAC Activity/Inhibition Assay Kit (EpiGentek) according to the manufacturer’s protocols, respectively, as done previously (13, 18). The enzymatic activities of HDACs were colorimetrically demonstrated and detected by an Epoch Microplate Spectrophotometer at 450 nm.

Global histone H3 acetylation quantification
Histone proteins from mouse breast tumors control and prenatal/maternal BSp treatment groups were extracted. Acetyl histone H3K9 (H3K9ac) and H3K14 (H3K14ac) were selected as high cooccurrence gene activation markers. The global histone H3K9 and H3K14 acetylation levels were detected according to the manufacturer’s protocol of EpiQuik Global Acetyl H3K9/H3K14 Quantification Kits (EpiGentek) by an Epoch Microplate Spectrophotometer at 450 nm.

Global DNA methylation analysis
The MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (EpiGentek) was used to quantify global DNA methylation status by specifically measuring levels of 5-methylcytosine (5-mC). DNA was extracted from mouse breast tumor tissues by DNeasy Blood & Tissue Kit (Qiagen). The 5-mC level was detected according to the manufacturer’s protocol and colorimetrically demonstrated by an Epoch Microplate Spectrophotometer at 450 nm.

RNA sequencing analysis
Next-generation sequencing was performed on the transcriptome using Illumina Platforms. mRNA sequencing was performed on the Illumina NextSeq500 as described by the manufacturer (Illumina Inc.). Briefly, the quality of the total RNA was assessed using the Agilent 2100 Bioanalyzer. RNA with an RNA integrity number of 7.0 or above was used for sequencing library preparation and Agilent SureSelect Strand Specific mRNA Library Kit was used as per the manufacturer’s instructions (Agilent). Library construction began with two rounds of polyA selection using oligo dT containing magnetic beads. The resulting mRNA was randomly fragmented with cations and heat, which was followed by first-strand synthesis using random primers with inclusion of Actinomycin D (2.4 ng/μL final concentration). Second-strand cDNA production was conducted with standard techniques, and the ends of the resulting cDNA were made blunt, A-tailed, and

Cancer Prev Res; 11(8) August 2018
Cancer Prevention Research

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adaptors ligated for indexing to allow for multiplexing during sequencing. The cDNA libraries were quantitated using qPCR in a Roche LightCycler 480 with the Kapa Biosystems Kit for Illumina library quantitation (Kapa Biosystems) prior to cluster generation. Cluster generation was performed according to the manufacturer’s recommendations for onboard clustering (Illumina). The raw RNA-Seq fastq reads were imported into CLC Genomics Workbench and aligned to the mouse GRCm38/mm10 reference sequence. Total transcript reads per kilo base pair per million (RPKM) were used as gene expression values. We utilized the R/Bioconductor package DESeq to evaluate differential gene expression for sequence count data by the use of negative binomial distribution (23). We tested for differential expression for each gene in the SV40 mouse offspring malignant mammary tumors after accounting for prenatal/maternal BSp treatment effect and used the Benjamini and Hochberg method to correct the \( P \) value for FDR. Gene functional associations by gene ontology were analyzed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources (https://david.ncifcrf.gov/). Genes showing significantly changed mRNA expression levels (Supplementary Fig. S2) were evaluated by Ingenuity Pathway Analysis (IPA) online software (Qiagen). Canonical pathway analysis utilizing the IPA library of canonical pathways identified the signaling routes that contained the significantly differentially expressed genes in the input dataset. \( P \) value was calculated using Fisher exact test determining the probability of the association between the genes in the dataset and the canonical pathway. The original RNA sequencing data have been deposited in Gene Expression Omnibus (GEO) with assigned GEO accession number GSE113900.

**Statistical analyses**

Statistical significance between the values of control and treatment groups was evaluated by one-way ANOVA followed by Tukey test for multiple comparisons. Statistical significance between the numbers of subjects with alternative outcomes was evaluated by \( \chi^2 \) and Fisher exact test by using GraphPad Prism 7.00 version. Values were presented as mean ± SD and \( P < 0.05 \) was considered statistically significant.

**Results**

**Prenatal/maternal BSp treatment resulted in maximal preventive and inhibitory effects on breast tumor development compared with postnatal BSp treatments**

In the current study, we chose to use the C3(1)-SV40 Tag (SV40) and Her2/neu transgenic mouse models that can develop breast tumors in their early lifespan that are driven by overexpression of the transgenes such as SV40 and Her2/neu oncogenes, respectively. These mouse models have been successfully applied in numerous breast cancer studies (22, 24, 25). In addition, the Her2/neu mouse model allows analyses of arising breast cancer that closely approximates human pathogenesis because Her2/neu gene activation is frequently seen in malignant human breast cancer. Thus, the use of two mouse models with different pathways of tumorigenesis further ensured the efficacy of the tested dietary plans. The dietary concentration for BSp used in the mouse studies was 26% BSp in formulated diet, which is equivalent to 266 g (~4 cups) BSp/per day for human consumption (13, 18, 26). Therefore, the concentration of BSp in this diet is physiologically available and represents a practical consumption level in the human diet. Prior to the experiment, we tested the potential influences of this prenatal/maternal BSp regimen on maternal and offspring health as well as mammary gland development in the offspring. Our results showed there was no negative effect of this dietary regimen on the abovementioned factors (data not shown), suggesting this diet is safe to use during pregnancy in vivo.

We initiated this study to determine the appropriate exposure window for dietary BSp diet that can maximize beneficial effects of this natural bioactive diet on prevention of human breast cancer and to explore the potential epigenetic mechanisms. As illustrated in Fig. 1, three well-designed dietary treatment plans were employed. This included prenatal/maternal, postnatal early-life, and adult BSp administrations, which represented the most conventional human dietary intake habits and also facilitated evaluation of optimal/critical exposure windows for the BSp diet–induced breast cancer early prevention effects.

In the prenatal/maternal treatment group in which BSp was administered during pregnancy and the lactation period, we found that the prenatal/maternal BSp diet can significantly decrease tumor incidence, inhibit early breast cancer development, and delay tumor latency during the entire course of experimentation in the offspring from two transgenic mouse models as illustrated in Fig. 2 (left). This result suggests a transplacental protective effect of the prenatal/maternal BSp diet on breast cancer prevention. To determine the optimal protective window for the BSp diet on breast cancer intervention, we also introduced two postnatal BSp treatment groups as parallel comparisons to eliminate maternal influences during early embryonic/fetal development. We subgrouped postnatal BSp treatments into early-life and adult treatments to further investigate the temporal impacts of the BSp diet on breast cancer development postnatally (Fig. 1). As expected, postnatal early-life BSp treatment beginning after weaning led to deceased tumor incidence and delayed tumor latency in both tested animal models, although the effects were not as profound as observed in the prenatal/maternal BSp treatment (Fig. 2, middle). However, adulthood-administered BSp diet did not reduce mammary tumorigenesis (Fig. 2, right), suggesting the importance of temporal factors for BSp exerting its chemopreventive effects.
Besides tumor incidence, tumor volume, and latency changes, we also observed other tumor-related phenotypic changes including tumor weight, pathologic/histologic appearance and metastasis status in response to our treatment plans. We found that neither of the treatments affected breast tumor pathologic/histologic appearance in both tested transgenic mouse models (Supplementary Fig. S3). We therefore compared the tumor prevention rate (tumor-free rate at the endpoint), inhibition rate (an index for tumor weight change), and metastasis rate including both local and remote metastasis as well as the ratio of extended tumor latency by dietary BSp administration during different lifetime points including prenatal/maternal, postnatal early-life, and postnatal adult exposures as summarized in Table 1. Our results showed that the prenatal/maternity BSp diet led to maximal and significant preventive and inhibitory effects on breast cancer development in both SV40 and Her2/neu transgenic breast cancer mouse models. Tumor latencies were significantly extended by 10.34% in SV40 and 30.83% in Her2/neu mice when the BSp diet was administered prenatally as compared with 4.9% and 18.8% if administered beginning postnatal early-life stage. In addition, early treatment with the BSp diet, especially during the prenatal/maternal stage, can decrease later-life breast tumor metastasis rate including direct invasion in adjacent lymph nodes and skeletal muscle (stage III) and remote metastasis such as lung metastasis (stage IV) as compared with the control, although no statistical significance was found, indicating this early dietary intervention may primarily affect tumor initiation process but not tumor progression process. However, there were no preventive or inhibitory effects observed if the BSp diet was administered later in life when the animals reached adulthood, indicating an exposure window sensitivity could be a key factor for BSp in breast cancer prevention. These results, for the first time, provide direct evidence of the temporal effects of dietary BSp exposure with respect to its efficacy on breast cancer prevention.

**Table 1.** Comparison of breast tumor growth between different temporal BSp treatment groups in two transgenic mouse models

<table>
<thead>
<tr>
<th>Animal experimental design</th>
<th>aPrevention rate (%) in SV40 mice</th>
<th>bInhibition rate (%) in SV40 mice</th>
<th>cRatio of extended tumor latency (%) in SV40 mice</th>
<th>dMetastasis rate (%) in SV40 mice</th>
<th>ePrevention rate (%) in Her2/neu mice</th>
<th>fInhibition rate (%) in Her2/neu mice</th>
<th>gRatio of extended tumor latency (%) in Her2/neu mice</th>
<th>hMetastasis rate (%) in Her2/neu mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>27.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Prenatal/maternal BSp</td>
<td>25%</td>
<td>56.9%</td>
<td>10.34%</td>
<td>10</td>
<td>50%</td>
<td>37.1%</td>
<td>30.83%</td>
<td>15</td>
</tr>
<tr>
<td>Postnatal early-life BSp</td>
<td>14.2</td>
<td>48.6%</td>
<td>4.9</td>
<td>14.2</td>
<td>20%</td>
<td>27.2%</td>
<td>18.8%</td>
<td>15</td>
</tr>
<tr>
<td>Postnatal adult BSp</td>
<td>10</td>
<td>3.57%</td>
<td>0.52</td>
<td>20</td>
<td>0</td>
<td>-6.38%</td>
<td>-2.95%</td>
<td>30</td>
</tr>
</tbody>
</table>

1^Inhibition rate (%) = tumor-free mice number/total mice number.

2^Ratio of extended tumor latency (%) = (mean tumor weight at sacrifice of the treatment group)/(mean tumor weight at sacrifice of the control group) x 100.

3^Metastasis cases include direct invasion such as adjacent lymph nodes and skeletal muscle (stage III) and remote metastasis such as lung metastasis (stage IV).

4^P < 0.05, significantly different from the control group.

5^P < 0.0001.
**Prenatal/maternal BSp diet led to gene expression changes in multiple epigenetic-controlled tumor-specific genes**

To explore the molecular mechanisms by which the prenatal/maternal BSp diet affected early breast carcinogenesis, we evaluated breast tumor samples derived from SV40 mice treated with the prenatal/maternal BSp diet. Our previous studies indicate that dietary SFN, the most abundant bioactive compound in BSp, can induce epigenetic reactivation of tumor suppressor genes and inhibition of tumor-promoting genes such as *hTERT* and *c-Myc* in human breast cancer cells (12, 18). We therefore evaluated gene expressions on several epigenetic-controlled key tumor-related genes such as *p53*, *p16INK4a*, *TERT* (telomerase reverse transcriptase), and *c-Myc* in the breast tumors of the offspring.

We found that the prenatal/maternal BSp diet induced significant increases of gene transcription in tumor suppressor genes such as *p53* and *p16INK4a* (Fig. 3A), and significantly decreased expressions of tumor-promoting genes such as *TERT* and *c-Myc* (Fig. 3B) in the offspring breast tumors compared with the control. In addition, significant protein level changes of these genes were consistent with gene transcription level changes shown in Fig. 3C–F. These gene expression changes were positively correlated to the effects of the prenatal/maternal BSp diet on interfering with breast cancer initiation. Moreover, the early-life BSp treatment was also effective in inducing key gene expression changes including the abovementioned tumor-related genes as well as the epigenetic-related genes (Supplementary Fig. S4), which are associated with its anticancer effects as compared with control and adult-BSp treatment. Because these key tumor-related genes are frequently regulated by epigenetic mechanisms, dietary components with epigenetic-regulatory properties may affect these gene expressions through regulation of epigenetic mechanisms leading to their chemoprevention effects against cancer (10–13, 27, 28). SFN in the BSp diet is believed to act as an epigenetic modulator to influence gene expression (10–13, 17, 18); it is speculated that the prenatal/maternal BSp diet may influence these gene expressions via a transplacental epigenetic regulation leading to a delayed tumor development in later life.

**Figure 3.**
Expression changes of specific epigenetic-controlled tumor-related genes by the prenatal/maternal BSp treatment. qRT-PCR and Western blot analysis were performed to measure gene expression of tumor suppressor genes such as *p53* and *p16* (A and C), and tumor-promoting genes such as *TERT* and *c-Myc* (B and D) in breast tumors of SV40 mouse offspring born to the mothers treated with the BSp diet. A and B, Relative gene transcription expression. C and D, Western gel blot showing protein levels. **β-Actin antibody was used to ensure equal loading. Representative photograph from an experiment was repeated three times.** E and F, Histogram of quantification of the protein levels. Data are in triplicate from three independent experiments and were normalized to GAPDH or **β-actin and calibrated to levels in untreated samples. Columns, mean; bars, SD. **·, *P* < 0.05; **··, *P* < 0.01; **···, *P* < 0.001, significantly different from control.**
Prenatal/maternal BSp diet may influence epigenetic pathways via regulation of HDAC1 and subsequent histone acetylation modification patterns

To further determine the epigenetic mechanisms, we assessed gene expressions and enzymatic activities of two important epigenetic-modulatory enzymes including HDAC1 involved in regulation of histone acetylation and DNA methyltransferase1 (Dnmt1) involved in regulation of DNA methylation processes in maternally BSp-treated breast tumors in SV40 mouse offspring. Our results showed that the prenatal/maternal BSp diet significantly decreased gene expression (Fig. 4A) and enzymatic activity (Fig. 4B) of HDAC1, but did not affect Dnmt1 gene expression. Further analysis showed that the prenatal/maternal BSp treatment can only slightly decrease global DNA methylation level by evaluating global 5-mC levels in the mouse breast tumors, and gene expressions of two important de novo DNA methyltransferases, Dnmt3a and Dnmt3b, were not affected as well (Supplementary Fig. S5). These results suggest that the prenatal/maternal BSp diet may primarily influence histone modification processes rather than DNA methylation processes that may contribute to its early breast cancer prevention effects.

Consistently, we observed increased histone acetylation levels in two important histone acetylation markers, histone acetyl-H3K9 and acetyl-H3K14, in response to the prenatal/maternal BSp treatment (Fig. 4C and D). Elevated levels of histone acetyl-H3K9 and acetyl-H3K14 are likely due to decreased HDAC1 expression that can lead to a more open and loose chromatin structure resulting in active transcriptional gene expression patterns, which may contribute to reactivation of tumor suppressor genes such as p16 and p53. However, suppression of tumor-promoting genes, TERT and c-Myc, may not be directly linked to downregulation of HDAC1 and increased histone acetylation. These results indicate that the prenatal/maternal BSp dietary regimen may exert its transplacental breast cancer chemoprevention effects through enhanced histone...
acetylation activator markers due to reduced HDAC1 expression and enzymatic activity.

**Differential gene transcription profiles induced by the prenatal/maternal BSp diet**

We believe many epigenetic-controlled genes were affected by the prenatal/maternal BSp treatment contributing to its early intervention in breast cancer initiation. We next sought to test global gene transcriptome changes by RNA sequencing analysis in the offspring breast tumors of SV40 transgenic mice to further identify key epigenetic-controlled genes in regulation of the prenatal/maternal BSp diet-mediated early breast cancer prevention. The original RNA sequencing data can be retrieved through an online data repository, GEO, with assigned GEO accession number GSE113900.

The hierarchical cluster analysis of moderate depth (20 million reads/sample) gene expression data showed differential transcriptome distribution in the breast tumors of mouse offspring between the control and prenatal/maternal BSp treatment groups (Fig. 5A). Our results showed that there were 1,558 significant differentially expressed genes identified (red dots in Fig. 5B) among 389,191 tested genes (Supplementary Fig. S2). Further comparison identified 1,390 genes with a fold change above 2 and a significant P value ($P < 0.05$) as illustrated in Fig. 5C. We also analyzed gene functional association by Gene Ontology analysis via DAVID in significantly changed transcriptome profiles in response to the prenatal/maternal BSp treatment. Our result indicates multiple cellular pathways have been regulated by the prenatal/maternal dietary BSp treatment, such as regulation of transcription, DNA repair, cell cycle, apoptosis, epigenetic pathways, and others (Fig. 5D). These pathway changes may contribute to the prenatal/maternal BSp diet–induced transplacental breast cancer prevention effects. Further IPA analysis (Fig. 5E) revealed that the "Role of BRCA1 in DNA Damage Response" signaling pathway had the highest P value among the 23 top canonical pathways in response to prenatal/maternal BSp diet. Because this signaling plays an important role in the initiation of breast tumorigenesis, it indicates that the BRCA1 signaling pathway could be an important molecular target for prenatal/maternal BSp diet-induced early chemopreventive effects on breast cancer.

**Discussion**

Cruciferous vegetables and their derived phytochemical extracts such as SFN and I3C have been well demonstrated to act as potent cancer chemopreventive products from various laboratory studies and clinical trials (www.clinicaltrials.gov; refs. 10–14, 18). Although many preclinical studies have reported efficacy, safety, pharmacokinetics, and molecular mechanisms for cruciferous vegetable-derived compounds, there are still many challenging questions remaining. In the current study, we confronted two important questions: (i) what is the temporal nature of a putative critical exposure window for cruciferous vegetables such as BSp exerting maximal cancer preventive effects; and (ii) do epigenetic mechanisms play a role in this process? We extended our hypothesis from conventional postnatal prevention models to a novel prenatal/maternal intervention model because it may represent a critical window sensitivity to various environmental factors such as diet. During these key stages in the lifespan, critical developmental events such as epigenetic programming, organismal differentiation, and maturation can be influenced by environmental and nutritional factors, which may determine disease risk later in life (2, 29).

Our results show a prenatal/maternal BSp diet exhibited maximal preventive effects on breast cancer development compared with the postnatal early-life treatment, which also showed protective effects although not as profoundly as observed in the prenatal/maternal treatment. Strikingly, the postnatal adult treatment did not show any preventive effect in two different breast cancer transgenic mouse models. These results illustrate the importance of temporal sensitivity that can affect chemoprevention potential of a bioactive dietary administration. Our results reveal a likelihood that the effectiveness of the BSp diet on breast cancer prevention may primarily depend on how early the individual has been exposed to this diet. It suggests that a dietary BSp regimen consumed during pregnancy/lactation or from postnatal early-life stage may render more efficacious effects on breast cancer prevention later in life than when consumed beginning from adulthood. Notably, the impact of time duration may be also important in influencing breast tumorigenesis because the postnatal adult BSp treatment with a shorter treatment period shows less preventive effect compared with the postnatal early-life BSp treatment with longer treatment time. This may be also due to the importance of a dietary intervention window that occurs during a critical oncogenic transition period, which is in early life for these two tested transgenic mouse models (24, 25). Determination of a critical oncogenic transition period could be complicated in humans, which may partially explain the controversial findings of the adult BSp treatment on breast cancer development in the tested mouse models as compared with the previous studies (14). Thus, long-term consumption of BSp diet is recommended to prevent cancers in humans. Our studies focusing on prenatal/maternal dietary intervention may also provide an exciting avenue in early prevention of breast cancer in humans.

A novel concept focusing on in utero chemoprevention has received increasing attention in cancer prevention studies (29). Williams’ laboratory has reported that I3C, abundant in Brussels sprouts and cabbage, supplemented to the maternal diet reduces carcinogen-induced T-cell lymphoblastic lymphoma mortality and decreases lung
tumor multiplicity in the surviving offspring, and that I3C is transplacentally bioavailable to the developing fetus and also increases offspring survival (20). Epidemiologic studies also show that Polish migrants who consume high amounts of SFN-enriched cabbage have low breast cancer incidence (19). However, there is no direct evidence linking the prenatal/maternal BSp diet to later-life breast tumor development. To our knowledge, our study is the first investigation to assess the temporal transplacental effects of dietary BSp on early breast cancer intervention, which may provide important insights on appropriate administration of this botanical supplement that maximize its beneficial effects and avoid potential adverse effects on disease prevention.

Figure 5.
Prenatal/maternal BSp diet–induced differential gene transcription profiles by RNA sequencing analysis. RNAs from the prenatal/maternal BSp diet–treated SV40 mouse offspring breast tumors were extracted and analyzed by RNA sequencing. A, Hierarchical cluster analysis of mRNA expression profiles. Genes were selected with significant changes (P < 0.05 and fold change > 2). Columns indicate individual mRNA expression values and rows correspond to different treatment groups. B, Scatter plots show sequencing reads (25 million reads/sample) in response to the prenatal/maternal BSp diet. Red dots represent individual genes with significant differential expression changes. C, Volcano plot shows fold variation and statistical significance of mRNA profiles among control and BSp treatment. The spots in cycles revealed the most significantly differentially expressed genes in each group compared with control group. D, Gene function association by gene ontology analysis via DAVID. y-axis showed multiples cellular pathways have been significantly regulated by the maternal dietary BSp treatment. Dotted line represented a threshold with a significant gene expression change (P < 0.05). E, Top canonical pathways by IPA analysis. The significantly differentially expressed genes were used for IPA analyses. Threshold criteria considered for the analysis are −log P value >1.3 or P < 0.05.
Temporal Dietary Epigenetics on Breast Cancer Prevention

Although a number of studies have reported a potential correlation of maternal BSp or SFN on preventive effects of different types of human diseases in the offspring (30, 31), precise mechanisms underlying these phenomena remain unknown. Previous studies have shown that multiple mechanisms and pathways may contribute to BSp or its derived phytochemicals such as SFN-induced robust prevention and therapeutic effects on various human cancers (14–16). Our studies and many others have recently discovered that epigenetic mechanisms may also play an important role during these processes (10–13, 17, 18). Cruciferous vegetables consumption as typified by the BSp diet is considered as an "epigenetics diet" that can modulate epigenetic pathways and reverse aberrant epigenetic markers leading to cancer-preventive and therapeutic effects (10). SFN as the most abundant and bioactive compound in the BSp diet has been identified as a potent HDAC inhibitor that preferably influences histone acetylation processes (17). These studies prompted us to address whether epigenetic mechanisms play a role on prenatal/maternal BSp-induced breast cancer prevention in later life. Epigenetic reprogramming plays critical roles during early embryogenesis (4). Nutritional exposure during this crucial time can alter epigenetic activities associated with disease-related genes and pathways, which may lead to different susceptibilities to diseases later in life (1–4). In this study, we tested four important tumor-related gene expressions, TERT, c-Myc, p16INK4a (p16), and p53 and found that these gene expression changes positively corresponded with the effects of the prenatal/maternal BSp diet on breast cancer inhibition. Because these genes are frequently regulated by epigenetic processes (27, 28), we speculate that epigenetic mechanisms may play a key role in influencing these gene expressions with respect to the temporal efficacy differences in response to the dietary BSp treatments.

We also found that the prenatal/maternal BSp diet significantly influenced gene expression and enzymatic activity of HDAC1, but did not detectably affect another important epigenetic modulator, DNA methyltransferases (Dnmt1, Dnmt3a, and Dnmt3b) as well as global DNA methylation level (5-mC), suggesting histone modifications may play a more important role on prenatal/maternal BSp-induced early chemoprevention effects than DNA methylation. This is consistent with the fact that SFN acts as a potent HDAC inhibitor in the BSp diet in regulation of histone modification processes (17). Further findings of increased enrichments of two important histone acetylation markers, histone acetyl-H3K9 and acetyl-H3K14, were consistent with the changes we observed in HDAC1 expression and enzymatic activity. Histone modification reprogramming mediated by changes in the specific epigenetic landscapes on core histones is critical to correct lineage specification and gene differentiation during early embryogenesis (4). Low protein maternal diet has been reported to influence specific key gene expressions in liver of the offspring via acetylation of histone H3 and H4 and methylation of H3K4 (32). Downregulation of HDAC1 and increased histone acetylation may also contribute to reactivation of tumor suppressor genes such as p16 and p53 in the mouse mammary tumors with prenatal/maternal BSp treatment. However, suppression of tumor-promoting genes, TERT and c-Myc, may be involved in other epigenetic mechanisms such as DNA methylation changes in the promoter regions. Further studies will facilitate the understanding of the precise epigenetic regulations including DNA methylation status and histone modification patterns in these individual target genes during different developmental stages. Our results indicate that an important regulatory mechanism of histone acetylation caused by the prenatal/maternal BSp diet could involve an active chromatin status leading to altered gene expression profiles in the offspring epigenome, which may eventually contribute to phenotypic changes such as disease susceptibility later in life.

Furthermore, our RNA sequencing analysis revealed differential gene expression changes in the transcriptome of the offspring breast tumors and multiple key signal pathways were significantly altered. Strikingly, further IPA analysis reveals that the BRCA1 signaling pathway is highly regulated and could serve as an important molecular target for prenatal/maternal BSp diet–induced early chemopreventive effects on breast cancer. BRCA1 and BRCA2 are well-studied genes linked to breast cancer risk (5). Studies have shown that BRCA1/2 germline mutations increase the risk of developing breast cancer, and they are also frequently regulated by epigenetic mechanisms. Our finding is very important because this could be the first study demonstrating the potential impact of in utero dietary BSp intervention on BRCA1/BRCA2 gene regulation, which may have profound impacts on early breast cancer prevention. Cho and colleagues have reported that a prenatal SFN diet can cause changes in utero signaling that profoundly regulates embryonic and extraembryonic tissue transcriptomics (33). Consistent with this report, our result suggests the transcriptome change may be governed by altered epigenomic profiles due to in utero exposure to the BSp diet. Advanced epigenetic array assays will be performed in our future studies that will facilitate understanding of the molecular epigenetic mechanisms and identify key epigenetic-controlled genes in regulation of prenatal/maternal BSp-mediated early breast cancer prevention.

The elucidation of the efficacy of early, in utero, chemoprevention of breast cancer using epigenetic dietary intervention is the most novel aspect of this investigation. Our study provides important implications for the efficient use of BSp during pregnancy and postnatal early life on prevention of breast cancer in later life, and potential
epigenetic mechanisms may be involved in this process. This study should have significance for potentially developing a novel dietary regimen for breast cancer prevention that may benefit human health. Future work will be necessary however to elucidate the precise molecular epigenetic pathways as well as to identify key epigenetic candidate genes altered by the BSp diet. Furthermore, clinical studies aimed to test the safety and effectiveness of this novel dietary regimen in human populations are also urgently needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Li, T.O. Tollefsbol
Development of methodology: Y. Li, T.O. Tollefsbol
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Li, S. Li, T.O. Tollefsbol
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Li, P. Buckhaults, T.O. Tollefsbol
Writing, review, and/or revision of the manuscript: Y. Li, T.O. Tollefsbol
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Li, S. Li, T.O. Tollefsbol
Study supervision: T.O. Tollefsbol

Acknowledgments

This work was supported by grants from the NIH R01 CA178441 (to T. Tollefsbol), R01 CA204346 (to T. Tollefsbol), and R01 AT009373 (to Y. Li) and the American Institute for Cancer Research AICR 316184 (to T. Tollefsbol).

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Received December 17, 2017; revised April 6, 2018; accepted May 7, 2018; published first May 15, 2018.

References

Cancer Prevention Research

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Yuanyuan Li, Phillip Buckhaults, Shizhao Li, et al.


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