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A presurgical-window intervention trial of isothiocyanate-rich broccoli sprout extract in patients with breast cancer

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Abstract

Scope: Dietary isothiocyanates (ITCs) from cruciferous vegetables have shown potent anti-breast cancer activities in preclinical models, but their anticancer effects *in vivo* in breast cancer patients remain elusive. A proof-of-principle, presurgical window of opportunity trial was conducted to assess the anticancer effects of dietary ITCs in breast cancer patients.

Methods and Results: Thirty postmenopausal breast cancer patients were randomly assigned to receive ITC-rich broccoli sprout extract (BSE) (200 μmol ITC/day) or a placebo for two weeks. Expression of biomarkers related to ITC's functions were measured in breast cancer tissue

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Author Contributions

L.T., Y.Z., S.M. Y.W. and C.A. conceptualized and designed the research. J.F. provided BSE. Z.W. and R.P. processed urine samples. C.T. and J.Q. performed urinary proteomics analysis. T.K. reviewed tissue slides. S.E. H.C. K.T. and J.Y. helped with participant identification and enrollment. Z. W. and L. T. analyzed, interpreted data, and wrote the manuscript. All authors reviewed the manuscript, commented, and approved the final manuscript.

Conflict of Interest

The Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

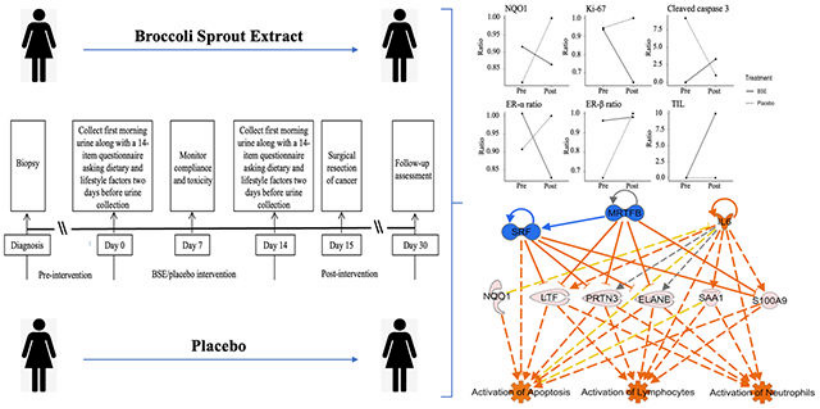
J.F. retired from the full-time faculty at Johns Hopkins University in 2020 and has consulted for food and supplement companies since then.

specimens at pre- and post-interventions using immunohistochemistry staining. First morning urine samples were collected at both timepoints for proteomic analysis. Overall, the study showed high compliance (100%) and low toxicity (no grade 4 adverse event). Trends of increase in cleaved caspase 3 and tumor-infiltrating lymphocytes (TILs) and trends of decrease in Ki-67 and nuclear to cytoplasm ratio of estrogen receptor (ER)-α were observed in the BSE arm only, consistent with the significantly altered signaling pathways identified in urinary proteomic analysis.

Conclusions: Anticancer activities of ITCs was observed in breast cancer patients, supporting the potential beneficial roles of ITC-containing cruciferous vegetables in breast cancer prognosis.

Graphical Abstract

Thirty postmenopausal breast cancer patients were randomly assigned to receive BSE or placebo for two weeks before surgery. Tissue and urine samples were collected at pre- and post-intervention. Trends of biomarker changes in breast cancer tissue aligned with significantly altered signaling pathways identified in urinary proteomic analysis, showing induction of apoptosis and modulation of immune function by BSE intervention.



Keywords

Breast cancer; Broccoli sprout extract; Chemoprevention; Intervention trial; Isothiocyanate

Introduction

Breast cancer is the most common cancer diagnosed among women in the U.S. (excluding skin cancers), and is also the second leading cause of cancer death among women after lung cancer [1]. As of January 2021, more than 3.8 million women have a history of breast cancer in the U.S. [2]. Although most breast cancer survivors are considered “cancer-free”, they are facing increased risks of disease recurrence and early death in comparison to the general population [3]. Breast cancer survivors often ask if there are additional strategies, beyond medical treatments, to help improve their cancer prognosis and survival. Studies have indicated that breast cancer patients are motivated to change their diet and/or take supplements after diagnosis [4–6]. Thus, a practical and promising strategy is to adapt a healthy lifestyle, especially with respect to diet. Cruciferous vegetable as a unique dietary

source of isothiocyanates (ITCs), a family of promising anticancer agents, is a potential means of improving breast cancer prognosis.

Mounting epidemiological studies have shown inverse associations of cruciferous vegetable intake with breast cancer risk [7], but few studies have examined their associations with survival outcomes, yet the findings were inconsistent [8]. The multi-faceted anticancer activities of dietary ITCs are well-documented in various preclinical cancer models including breast cancer [9]. Sulforaphane, the primary ITC from broccoli, is the most extensively studied ITC [10], showing induction of NAD(P)H quinone dehydrogenase 1 (NQO1) [11], inhibition of cell proliferation [12], induction of apoptotic cell death [13], and alteration of estrogen receptor (ER) signaling [13, 14] in *in vitro* and *in vivo* breast cancer models. However, data demonstrating anticancer effects of ITCs *in vivo* in breast cancer patients remain elusive. Such data could provide direct evidence to support dietary modification to improve breast cancer survivorship.

Leveraging the window between diagnosis and surgery for patients with newly diagnosed breast cancer, we conducted a two-week proof-of-principle intervention trial using a placebo-controlled, double-blinded, randomized design to assess the anticancer effects of dietary ITCs in breast cancer patients. ITC-rich broccoli sprout extract (BSE) was used to facilitate clinical translation [15], given its vegetable-base, its demonstrated safety profile [16, 17], and its higher content of sulforaphane as compared to mature broccoli [18]. An additional advantage of BSE is that release of ITCs from glucosinolates, the precursor of ITCs in cruciferous vegetables, is complete [18], thus avoiding inter-individual variability in ITC yields from the vegetables and allowing standardized ITC administration [19]. Tumor-infiltrating lymphocytes (TILs) and five biomarkers, including Ki-67, cleaved caspase 3, NQO1, ER-α and ER-β, were selected to evaluate the effect of ITCs on breast cancer using immuno-histochemistry staining (IHC). TILs, Ki-67 and cleaved caspase 3 are widely used as indicators of tumor immune microenvironment, cell proliferation, and apoptosis, respectively, in clinical trials [20–23]. NQO1, ER-α and ER-β are cellular targets of ITCs [11, 13, 14] and play important roles in breast cancer prognosis [24, 25]. In addition to tissue-level assessment, urinary proteomic analysis was performed to provide a global and holistic evaluation of signaling pathways altered by ITCs *in vivo* in breast cancer patients. The findings from this study provide first-hand data in breast cancer patients regarding the potential beneficial role of cruciferous vegetables in breast cancer and help begin a discussion about using ITC supplements in breast cancer survivors.

Experimental Section

Study design and participants recruitment

This was a proof-of-principle, short-term, placebo-controlled, randomized intervention study (clinicaltrials.gov NCT01753908). BSE or placebo was administered daily for two weeks immediately prior to scheduled surgical tumor resection. The intervention schedule did not alter or delay treatment. Participants were recruited from the breast clinic at Roswell Park Comprehensive Cancer Center (Roswell Park) between 2012-2018. Eligible participants were patients diagnosed with breast cancer at any stage, post-menopausal (to avoid the effect of menstrual cycles on certain biomarkers), had an Eastern Cooperative Oncology

Group (ECOG) performance status of ≤ 2, and had surgeries scheduled at least two weeks after confirmation of diagnosis. Women with any one or more of the following criteria were excluded from this study: prior invasive breast cancer, mastectomy or breast radiation within 12 months; treatment for any other malignancy within 12 months; use of reproductive hormone therapy within the last 90 days; intolerance to BSE taste, or being a current consumer of BSE; lack of willingness to avoid cruciferous vegetable intake during the two-week study period; current diagnosis of grade 3 or above gastroesophageal reflux disease (GERD), or disease states that interfere with digestion and absorption of BSE such as Crohn's disease, celiac sprue or other malabsorption syndromes. Breast surgeons identified eligible patients and then study coordinators consented patients for randomization. A total of 204 patients were screened for eligibility, of which 53 (26%) did not meet inclusion criteria, 47 (23%) were deemed inappropriate for the study at the discretion of the physician due to other comorbidities or treatment regimens, 73 (36%) declined for participation, and one patient withdrew after randomization due to intolerance to the BSE taste (Supplemental Figure S1). A total of 30 patients completed the study and were included in the analysis. Informed consent was obtained from all participants. The research protocol was approved by the Institutional Review Board at Roswell Park.

Dosage information

Enrolled participants were randomly assigned to the BSE or placebo arm based on a randomization log that was generated by the study statistician using a randomized permuted block method. Participants, clinical and research staff were blinded to the assignment of treatment arms. For the intervention arm, participants were given 14 doses of BSE powder containing approximate 200 μmol ITCs, equivalent to about 500g of fresh broccoli per day [26]. This dose of ITC-BSE has been evaluated in clinical trials, demonstrating safety and tolerance [11, 16, 17, 27]. The BSE was obtained from Johns Hopkins University under an IND (#117001) approved by the Food and Drug Administration. The extract was made from three-day-old broccoli sprouts with daikon myrosinase added post-extraction and cooling, to convert glucosinolates into ITCs, and then filtered, freeze-dried, and stored at −80° before aliquoting and distribution by the Investigational Drug Service (IDS). For the placebo arm, the same amount of plant-based microcrystalline cellulose (Spectrum Chemical MFG Corp., New Brunswick, NJ) was aliquoted and distributed by the IDS using identical bottles and labels. This product has been widely used as an inert substance in pill and tablet formulations and used as placebo in clinical trials. A 14-day supply of mango juice was distributed along with the BSE or placebo. Participants were instructed to mix the BSE or placebo powder with the supplied mango juice immediately prior to daily consumption to disguise the taste of ITCs, i.e., the unique pungent taste of cruciferous vegetables. Each participant was given a study calendar to keep track of dosing, side effects, and other medications/supplements. The study coordinators contacted the patient on day 7 (+/− 2 days) to assess compliance and/or concerns about the study.

Each participant provided a first morning urine sample at the beginning (day 0) and at the end of intervention (day 14), along with a brief 14-item questionnaire to collect information on dietary and lifestyle factors for the two days prior to the urine collection, including consumption of ITC-containing cruciferous vegetables and/or condiments,

smoking, drinking alcohol or use of antibiotics that could affect microflora thus influence digestion and absorption of ITCs. Participants underwent cancer surgical resection on day 15 and were followed on day 30 (±3 days) to assess any intervention-related toxicities. Sections from the Formalin-Fixed Paraffin-Embedded (FFPE) biopsy at pre-intervention and surgical breast resection specimen at post-intervention were requested from the Pathology Network Shared Resources (PNSR) at Roswell Park. The study design is presented in supplemental Figure S2.

Urinary measurement of ITC metabolites

Urinary levels of ITC metabolites were measured to monitor study compliance using a High-Performance Liquid Chromatography (HPLC)-based cyclocondensation assay [28]. The method measures total ITCs and their metabolites *via* the reaction of their characteristic structure of the –N=C=S group with the thiol group of 1,2-benzenedithiol to form a 1,3-benzodithiole-2-thione, which is eluted at 5-6 min at 365 nm using HPLC [29]. Briefly, in a 1 mL reaction (adjusted with water), each sample (up to 100 μL) was mixed with 400 μL methanol (Thermo Fisher Scientific, San Jose, CA), 250 μL of 500 mM sodium borate buffer (pH 9.25) and 100 μL of 1,2-benzenedithiol (Thermo Fisher Scientific, San Jose, CA) at the concentration of 1.24 g/mL in methanol in a 4-mL glass vial with a screw cap. The reaction mixture was incubated at 65°C for 2 h and centrifuged at low speed for 10 min. A total of 300 μL of supernatant was loaded into an autosampler and analyzed by HPLC. An Agilent HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a model G1311B pump, a model G1329B autosampler, a model G1315C photodiode array detector, and an Agilent Chemostation chromatography data system were coupled to an analytical C18 reverse-phase column (HiCHROM, Partisil 10 μm ODS-2, 250 x 9.4 mm) for the cyclocondensation assay. The mobile phase consisted of methanol (80%) and H_2O (20%) running at a flow rate of 1.75 mL/min with a sample injection volume of 100 μL and a detection wavelength at 365 nm. Blank controls and a series of sulforaphane standards (LKT Laboratories, St Paul, MN) were included in each run. Duplicate reactions were performed for each sample and a coefficient of variation (CV) was calculated. Any samples with CV > 10% were repeated. Urinary creatinine levels were measured using a Cayman creatinine assay kit (Ann Arbor, MI) and were used for normalization of urinary ITC levels.

Biomarker staining and quantification

Six 5-μm tissue sections were obtained from the FFPE specimen (biopsy and surgical resection) and subjected to hematoxylin and eosin (H&E) staining for evaluation of TILs and IHC staining of Ki67, cleaved caspase 3, NQO1, ER-α and ER-β, respectively, at the PNSR. Except for ER-β, the Dako Omnis autostainer system (Agilent Technologies, Santa Clara, CA) was used with deparaffinization using Clearify (American Mastertech; catalog #CACLEGAL) and target retrieval for Ki67 and ER-α using Flex TRS Low (Dako, GV805) and for cleaved caspase 3 and NQO1 using Flex TRS High pH (Dako, GV804), respectively. Antibodies for Ki67 (Dako, M7240 at 1/100 dilution), cleaved caspase 3 (Cell Signaling, #9661 at 1/50 dilution), NQO1 (Affinity Bioreagents, MA1-16672 at 1/1250 dilution), and ER-α (Santa Cruz, sc-543 at 1/100 dilution) were applied, respectively, and followed by Flex Rabbit Linker (Dako, GV80911-2) and Flex horseradish peroxidase polymer (Dako, DM843). For ER-β, slides were deparaffinized through a series of xylenes and graded

alcohols, pretreated in high pH buffer (Agilent, S2367), quenched in 0.3% H_2O_2, then followed by protein block (Dako, X0909) before loading onto the Dako Autostainer system. ER-β antibody (R&D Systems, PP-PPZ0506-00 at 1/100 dilution) was applied and followed by mouse Envision (Agilent, K4001). Slides were stained with diaminobenzidine (Dako, K3468) and counterstained with hematoxylin for visualization. Relevant non-immune rabbit or mouse IgG was used as negative control.

Digital images of the stained sections were acquired using an Aperio ScanScope XT and captured using Aperio Digital Image Analysis Software (Leica Biosystems Inc, Lincolnshire, IL). H&E staining was reviewed first to confirm tumor grade and stage. TILs between nests of invasive carcinoma and at the invasive edge but not intratumorally were evaluated [23] and scored following the International TILs Working Group 2014 to estimate the percentage of TILs in stroma [30]. Neutrophils and eosinophils were not scored. For IHC staining, breast cancer areas were circled manually and scored using algorithms optimized for each antibody at the PNSR. All evaluations were performed under the supervision of the study pathologist (Khoury T.). H-scores were derived from the staining intensity (0, 1, 2, 3) multiplied by the percentage of positive cells for each intensity category. Given the differences of the biomarkers in cellular localization, H-scores were generated for cytoplasm staining of NQO1, nucleus staining of Ki67, nuclear and cytoplasm staining separately for ER-α and ER-β; while the number of positive cells per area (mm^2) was used for cleaved caspase 3. For ER-α and ER-β, the sum of nuclear and cytoplasm expression as well as the ratio of nuclear to cytoplasm expression was calculated as indicators of ER status, since activation of ER signaling requires nuclear translocation of receptors from cytoplasm [31].

Urinary proteomics analysis

Urine samples (10 mL) were centrifuged at 4,500 x g for 20 min at 4 °C, and then the supernatant was collected and pelleted with the addition of 40 mL chilled acetone with continuous vortex at −20 °C overnight. After removal of the supernatant, the pellet was extracted with detergent in the lysis buffer (50 mM Tris-formic acid, 150 mM NaCl, 0.5% sodium deoxycholate, 2% SDS, 2% NP40, pH 8.0) and sonicated on ice (Qsonica, Newtown, CT). After centrifugation at 18,000 × g for 30 min at 4 °C, the supernatant was transferred, and the protein concentration was determined by a Compat-Able bicinchoninic acid protein assay kit (Thermo Fisher Scientific, San Jose, CA). Samples containing 100 μg protein were reduced by the addition of 5 mM dithiothreitol for 30 minutes, followed by alkylation using 40 mM iodoacetamide for 30 minutes at 37 °C in darkness. The protein mixture was precipitated by stepwise addition of 5 volumes of cold acetone with continuous vortex and then incubated at −20 °C overnight. After centrifugation at 18,000 g for 30 min at 4 °C, the pellets were collected and washed using an 85/15% (v/v) chilled acetone/water solution, and then partially air-dried. The pellets were resuspended with 100 μL buffer (50 mM Tris-formic acid, pH 8.5) and digested using trypsin at a 1:20 (w/w) enzyme/substrate ratio. The mixture was incubated at 37 °C overnight with constant mixing at 200 RPM. A 1% (v/v) final concentration of formic acid was added to terminate digestion.

The digested protein samples were subjected to the Nanoscale Liquid Chromatography coupled to Tandem Mass Spectrometry (Nano-LC-MS/MS) analysis. A Thermo Scientific

UltiMate 3000 RSLCnano system and an Orbitrap Fusion Lumos mass spectrometry (San Jose, CA) were employed. The mobile phase A contained 2% acetonitrile and mobile phase B contained 88% acetonitrile, each in water with 0.1% formic acid. The nano-LC column was heated at 52 °C. Four μL of digested mixtures were loaded onto a large-ID trap (300 μm ID x0.5 cm, packed with Zorbax 3-μm C18 material) with 1% B at a flow rate of 10 μL/min for 3 min. The trapped peptides were then back-flushed onto the nano-LC column (75 μm ID x 60 cm, packed with Waters XSelect CSH 2.5-μm C18 material) at a flow rate of 250 nL/min. A 180-min gradient was used and the optimized gradient profile was as follows: 4% over 3 min; 4 to 11% over 5 min; 11 to 28% over 117 min; 28 to 50% over 10 min; 50 to 97% over 1 min and isocratic for 17 min; and finally isocratic at 4% of B for 27 min. Mass spectrometry (MS) data were acquired under data-dependent product ion scan mode with a cycle time of 3 s. One scan cycle included a survey scan (m/z 400-1500) at a resolution of 240,000 with an AGC target of 5×10^5 and a maximum injection time of 50 ms. Ions with charge states between 2-7 were filtered for MS2 analysis. MS2 was performed by isolation at 1.2 Thomson with the quadrupole for high energy collision dissociation (HCD) fragmentation and detected by Orbitrap at a resolution of 15,000 with an AGC target of 5×10^4. The maximum injection time was 50 ms and the collision energy was 30%. Dynamic exclusion was enabled with a repeat count of 1 and an exclusion duration of 45 s.

Statistical analysis

Demographic and tumor characteristics of the participants were compared between the BSE and placebo arms for categorical variables using Pearson's Chi-square or Fisher's exact test if numbers in any individual cells were less than 5, and for continuous variables using a student t-test or Kruskal-Wallis test if data were not normally distributed. Given the severely skewed tissue-level data from staining, the Wilcoxon sign-rank test was used for comparisons between pre- and post-intervention within each arm first; then post-to-pre-intervention changes were calculated and compared between treatment arms using an exact Wilcoxon rank-sum test. To evaluate the overall trend of expression changes in biomarkers between pre- and post-intervention, the median of expression in post-intervention group of the placebo arm (placebo post) was used as the reference and the ratios of the medians of expression in the other three groups to the reference group (BSE pre, BSE post, and placebo pre) were calculated and presented for each specific biomarker. All the analyses were carried out using R version 3.6 (or higher) statistical software.

The MS raw data files were searched against the human protein database using the Proteome Discoverer v2.4 (Thermo Fisher Scientific, San Jose, CA) for protein identification. The search parameters were set as follows: 10 ppm tolerance for precursor ion mass and 0.02 Da for fragment ion mass with fully tryptic peptides restraint and a maximum of two missed cleavages. Static carbamidomethylation of cysteine, dynamic oxidation of methionine and n-terminal acetylation were used. The false discovery rate (FDR) was detected by the incorporated module Percolator in Proteome Discoverer using a target-decoy search strategy. The FDR 1% at peptide level and 1% at protein level were applied in this study. The protein quantification was performed by the Precursor Ions Quantifier module in Proteome Discoverer. The relative expression ratio was calculated by the average precursor ion intensities in each group. A z-score transformation was performed before performing

the hierarchical clustering and generating heatmaps using Proteome Discoverer. Euclidean distance similarity metric and complete linkage method were applied. Paired *t*-test was used to evaluate the difference between pre- and post-intervention in each arm with controlling multiple comparisons by FDR. Significantly altered proteins were subjected to Ingenuity pathway analysis (IPA, version 01-16, QIAGEN, Germany) to identify canonical pathways and regulator effects. IPA assigns scores for each pathway according to hypergeometric distribution, where the −log (P-value) is generated using Fisher's exact test and the value >1.3 (i.e., $P < 0.05$) was used as the cutoff threshold [32]. Consistency score, an indicator to describe the causal consistency of the upstream regulatory factor in the network and the dataset and dense connection metric between disease and function, was calculated for each potential regulator, with a higher score indicating more accuracy [32]. The predicted effect network was generated using regulators with top consistency scores.

Results

Patient characteristics

The demographic and clinical characteristics of study participants are summarized in Table 1. The study participants were primarily European American (97%), had an average age of 61 years at diagnosis, and showed a high proportion of being overweight/obese (80%). The majority of participants had early stages of invasive disease (84% stages I or II), with predominantly ER positive (90%), PR positive (80%), and HER2 negative (90%). Disease characteristics were distributed similarly between the BSE and the placebo arms. There was also no significant difference in lifestyle behaviors between the two arms at baseline, including smoking, alcohol consumption and use of antibiotics, despite a slightly higher proportion of participants reported consumption of ITC-containing cruciferous vegetables and/or condiments in the BSE arm in comparison to the placebo arm (40% versus 20%, $P = 0.43$) (Table 2). When lifestyle behaviors were compared between pre- and post-intervention (Table 2), a slight increase in the proportion of participants reporting alcohol use was observed in the placebo arm (of 15 participants, 4 at pre to 6 at post). The percentage of participants reporting consumption of ITC-containing food was reduced from 20-40% at pre-intervention to zero at post-intervention in both arms, in agreement with the study instruction not to consume any ITC-containing food during intervention. Given the potential impact of these lifestyle behaviors on biomarkers of interest, sensitivity analyses were conducted among the participants who did not report any of these lifestyle behaviors at both pre- and post-intervention, from which similar results were obtained. Therefore, the results based on all participants were presented.

Compliance and Toxicity

To monitor study compliance, urinary levels of ITC metabolites at pre- and post-intervention were compared between the BSE and the placebo arms (Table 3). At pre-intervention, the urinary level of ITC metabolites in the BSE arm was slightly higher than that in the placebo arm, consistent with the slightly higher proportion of participants reporting consuming ITC-containing food in the BSE arm (Table 2). As expected, the urinary level of ITC metabolites at post-intervention was over 200-fold higher in the BSE arm than in the placebo arm (228.0 versus 1.3 μmol/g creatinine, $P < 0.001$). Indeed, all participants in the BSE arm showed

increases in the urinary level of ITC metabolites, though ranges of net changes between post- and pre-intervention were wide, from 6.6 to 598.7 μmol/g creatinine. This is in sharp contrast to either the decreases or lack of changes in the placebo arm, indicating excellent study compliance.

A range of adverse events were reported by the study participants in both arms (Table 3). The total number of events was higher in the BSE arm than in the placebo arm (27 versus 13 events); however, 17 out of 27 events (63%) in the BSE arm were gastrointestinal system related, such as abdominal distention, diarrhea, dyspepsia, eructation, flatulence, nausea, as commonly reported symptoms from consumption of cruciferous vegetables. All other events were distributed similarly across two arms. There was no grade ≥ 4 adverse event reported in the study. The BSE and placebo arms had similar distribution of maximal grade of reported adverse events.

Changes in tissue-level biomarkers

The expression of five biomarkers, including NQO1, Ki67, ER-α, ER-β and cleaved caspase 3, and the percentage of TILs in breast cancer tumor tissue were compared between pre- and post-intervention separately in the BSE and the placebo arms, and then the post-pre expression changes were compared between the two arms (Table 4). All biomarkers displayed large interindividual variations. Overall, no significant difference in biker expression was observed either between pre- and post-intervention or between the BSE and placebo arms. However, the BSE arm showed an opposite direction of biomarker changes in comparison to the placebo arm, with trends of decrease in NQO1, Ki-67 and ER-α nuclear to cytoplasm ratio (the indicator of ER activation), trends of increase in cleaved caspase 3 and TILs, and almost no changes in ER-β nuclear to cytoplasm ratio (Fig. 1). In addition, large differences in baseline expression of NQO1, ER-α, ER-β, and cleaved caspase 3 were observed between the BSE and placebo arms, though the differences were not statistically significant due to the large interindividual variations.

Alterations of urinary proteomic profiles

A total of 2,061 proteins were identified in the urine, which showed diverse expression patterns across the four groups: pre- and post-intervention group of the BSE arm and pre- and post-intervention group of the placebo arm (Fig. 2A). When the urinary proteomic profiles were compared between pre- and post-intervention, a total of 178 proteins changed significantly after intervention in the BSE arm and 229 in the placebo arm (Fig. 2B). Between the arms, 62 proteins overlapped, leaving a panel of 116 proteins specifically altered by the BSE intervention, of which 59 proteins were upregulated and 57 were downregulated. This BSE-specific panel of proteins was subjected to pathway analysis using IPA, showing 38 significantly enriched canonical pathways from upregulated proteins and 17 pathways from downregulated proteins (Tables S1 & S2). The top pathways with a −log(P-value) > 2 are presented in Fig. 2C. The ‘Acute phase response’ signaling was the highest-ranking signaling pathway, followed by pathways related to ITC metabolism/functions or pathways that are known to play a role in breast tumorigenesis, such as ‘LXR/RXR activation’, ‘FXR/RXR activation’, ‘Superoxide radicals degradation’, ‘NRF2-medidated oxidative stress response’, ‘Glutathione-mediated detoxification’, ‘Mitochondrial

dysfunction', 'Inhibition of matrix metalloproteases', and others. Furthermore, regulator effect analysis was conducted using IPA to predict the potential upstream regulatory networks and downstream functions based on the proteins specifically altered by the BSE intervention (Fig. 2D). The top predicted network contained three upstream regulators (serum response factor, SRF; interleukin 6, IL-6; and myocardin related transcription factor B, MRTFB) and three downstream functions (induction of apoptosis, activation of lymphocytes, and activation of neutrophils), and was mediated through six BSE-altered proteins, including NQO1, proteinase 3 (PRTN3), S100 calcium binding protein A9 (S100A9), lactotransferrin (LTF), serum amyloid A1 (SAA1), and neutrophil elastase (ELANE).

Discussion

In this randomized pilot intervention study, we evaluated the effect of ITCs in breast cancer patients using ITC-rich BSE. The daily consumption of 200 μmol ITC in BSE for two weeks, equivalent to about 500 g of fresh broccoli per day [26], was well tolerated. Excellent compliance with and low toxicity of the BSE were observed (Table 2 and 3). Although biomarker changes at the breast cancer tissue level were not statistically significant between pre- and post-intervention, general trends of increase in cleaved caspase 3 and TILs and decrease in Ki-67 and ER-α nuclear to cytoplasm ratio were observed in the BSE arm, supporting ITC-induced activation of apoptosis and immune function but inhibition of ER-α signaling and cell proliferation (Table 4 and Fig. 1). These tissue-level effects observed in the BSE arm were confirmed by global evaluation of urinary proteomic profiles between pre- and post-intervention (Fig. 2). A total of 116 urinary proteins were altered specifically by the BSE intervention involving 55 enriched signaling pathways, of which multiple pathways were known related to ITC functions, such as 'Superoxide radicals degradation' and 'Nrf2-mediated oxidative stress response'. Importantly, the top predicted network based on these altered urinary proteomic profiles indicated activation of three downstream functions: induction of apoptosis, activation of lymphocytes and activation of neutrophils. The changes in specific biomarkers at the breast cancer tissue level and the changes in global proteomic profiles at the individual level are highly concordant to support anticancer effects of ITCs on breasts cancer. Further, these findings are consistent with the anticancer mechanisms of ITCs identified in *in vitro* and *in vivo* studies [9–14].

ITCs, including sulforaphane, the primary ITC in BSE, showed repression of ER-α signaling pathways, regulation of immune function, inhibition of cell proliferation, and induction of apoptosis in human breast cancer cell lines [10–14]. Similar effects were observed in breast cancer patients at the breast cancer tissue level, showing trends of decrease in Ki67 and ER-α nuclear to cytoplasm ratio and increase in cleaved caspase 3 and TILs in the BSE arm, in comparison to the opposite direction of changes in the placebo arm. ITCs also showed upregulation of ER-β expression in human mammary cells, but the effect was only observed in benign breast epithelial cells, not in breast cancer cells [33]. This finding agrees with our observation of little or no changes in ER-β expression at the breast cancer tissue level by BSE intervention. Of all examined tissue-level biomarkers, NQO1 is the only one showing an unexpected direction of change: a trend of decrease in the BSE arm but increase in the placebo arm (Fig. 1). ITCs are well known inducers of NQO1 due

to their activation of the NRF2-antiodidant responsive element (ARE) signaling pathway [34]. Indeed, urinary proteomic analysis revealed activation of NRF2 signaling pathway and induction of NQO1 globally in the BSE arm (Fig. 2C and 2D). Lack of corresponding changes in NQO1 expression at the breast cancer tissue level by the BSE could relate to the fact that large proportion of participants in the BSE arm had high baseline expression level of NQO1 in comparison to that in the placebo arm, while individuals with high-baseline NQO1 levels generally had a decrease in NQO1 expression at post-intervention regardless of the BSE or placebo arms. We did observe a general trend of increase in NQO1 by BSE among individuals with baseline expression level below the medium. Overall, the effects of ITCs observed in preclinical models are recapitulated in breast cancer patients in clinical intervention trial settings.

Evaluation of the global effect of BSE intervention in breast cancer patients by urinary proteomic analysis further supports the potential beneficial role of ITCs against breast cancer. First, changes in urinary proteomic profiles agree with the trends of tissue-level biomarker changes in the BSE arm. Based on more than 100 urinary proteins altered specifically by BSE intervention, the top predicted networks indicated induction of apoptosis and activation of lymphocytes and neutrophils, which are in line with the increase of cleaved caspase 3 and TILs observed at the breast cancer tissue level. Second, urinary proteomic analysis showed modulation of multiple signaling pathways by BSE intervention, supporting multi-faceted anticancer mechanisms of ITCs identified in preclinical studies. One of the well-recognized mechanisms underlying ITCs cancer protective effect is activation of the NRF2 signaling pathway, which upregulates expression of genes involved in cellular defense by detoxifying exogenous carcinogens and endogenous reactive oxygen species (ROS) [35]. Multiple upregulated signaling pathways belong to this category, including ‘NRF2-mediated oxidative stress response’, ‘Glutathione-mediated detoxification’, ‘Superoxide radicals degradation’, ‘Production of NO and ROS in macrophages’ and pathways related to xenobiotic metabolism, such as ‘Xenobiotic metabolism AHR signaling’, ‘Xenobiotic metabolism general signaling’, ‘Xenobiotic metabolic signaling’, and ‘Aryl hydrocarbon receptor signaling’ (Fig. 2C). In addition, ITCs have shown targeting on mitochondria to induce apoptosis [36] and downregulating matrix metalloproteases to inhibit cancer metastasis [37] in cancer cell models, which aligns with the alteration of ‘Mitochondrial dysfunction’ and ‘Inhibition of matrix metalloproteases’ signaling pathways by BSE intervention. Lastly, pathway analysis based on altered urinary proteomic profiles revealed additional molecular targets of ITCs, supporting the potential role of ITCs in prevention of breast cancer recurrence and progression. Activation of liver X receptor (LXR) and farnesoid X receptor (FXR) signaling pathways were observed by BSE intervention (Fig. 2C). Both LXR and FXR are nuclear receptors that regulate a variety of metabolic enzymes and transporters to control homeostasis of estrogen and cholesterol/bile acid, respectively [38–40]. Activation of LXR leads to inhibition of estrogen-dependent breast cancer growth [41], while activation of FXR inhibits breast cancer cell proliferation and induces apoptosis [42].

Network analysis further predicted three upstream regulators of ITCs, SRF, MRTFB, and IL-6, along with three downstream effects of ITCs, induction of apoptosis, activation of lymphocytes and activation of neutrophils. Importantly, the validity of the predicted network

was supported by extensive evidence from *in vitro* and *in vivo* studies of ITCs in cancers. SRF induces self-renewal of tumor-initiating breast cancer cells [43] and promotes cancer metastasis [44]. MRTFB is a key coactivator of SRF and plays a role in breast cancer metastasis in both SRF-dependent and SRF-independent ways [45]. SRF and MRTFB are transcriptional factors, and their activities rely on subcellular localization, translocation and dimerization in response to Rho A/Rock and ERK/MAPK signaling pathways [46]; while both signaling pathways are well known targets of ITCs [47–49]. Therefore, ITCs are unlikely to alter the expression level of SRF and MRTFB; instead, ITCs affect transcriptional activities of SRF and MRTFB through modulation of respective signaling pathways as shown in cancer cell models [50, 51]. On the other hand, modulation of IL-6 expression in cancer has been widely reported by ITCs directly or indirectly via NRF2-ARE and/or NF-κB signaling pathways [52, 53]. Cytokine IL-6 regulates systemic inflammatory response during acute stimulation [54], corresponding to the activation of 'Acute phase response signaling' in the BSE arm. In breast cancer, IL-6 signaling plays important roles in controlling breast cancer cell growth, metastasis, and cancer stem cell self-renewal [55]. Overall, the functions of these upstream regulators align with their downstream effects on apoptosis and immune cells and are supported by the changes in signaling pathways and molecular mediators following BSE intervention.

Besides induction of apoptosis, one of the well-documented anticancer mechanisms of ITCs as discussed above [12], immunomodulating effects of ITCs, in particularly sulforaphane (the principal ITC in the BSE), have been increasingly recognized in settings against aging, autoimmune diseases and cancers [56, 57] . Inhibition of anti-inflammatory cytokines and stimulation of immune responses via NRF2-ARE and NF-κB signaling pathways by ITCs have been widely reported [56]. In cancer cell models, sulforaphane activated Natural Killer cells and increased infiltration of B- and T-lymphocytes in tumors, resulting in enhanced immune responses and reduction of metastasis [58]. Interestingly, sulforaphane was reported to act as a pro-oxidant to inhibit inflammatory responses of primary human T-cells, raising a concern on potential interfering chimeric antigen receptor T (CAR-T) cell-based immunotherapy [59]. However, this concern is largely mitigated by a recent report that sulforaphane improves cytotoxicity of CAR-T cell by modulating the expression of programmed cell death 1 and ligand 1 (PD-1/PD-L1) [60]. Further investigations of the immunomodulating roles of ITCs in cancer immunotherapy are warranted.

Two lessons were learned from this pilot intervention trial. First, a relatively large sample size is required to accommodate wide interindividual variations that were observed in biomarker expressions (Table 4) as well as in urinary ITC metabolite levels (6.6 to 598.7 μmol/g creatinine in the BSE arm, Table 3). In the study, participants were instructed to consume the BSE in the evening and record the time of the first-morning urine collection, reducing the variations introduced by the time interval between the BSE consumption and the urine collection. Further, the BSE has the completed conversion of glucosinolates to ITCs, minimizing the influence of gut microflora on ITC production. Therefore, the wide range of urinary ITC metabolite levels in the BSE arm may at least partly reflect interindividual variations in ITC metabolism. ITCs are metabolized in humans primarily *via* the mercapturic acid pathway, in which glutathione S-transferase (GST) and N-acetyltransferase (NAT) are polymorphic with various isoforms showing different

efficiency of catalyzing ITCs [61, 62]. Even with administration of the same ITC dosage, ITC concentration at the breast cancer tissue level could vary among individuals due to the genetic variations in ITC-metabolism enzymes, contributing to variations in biomarker response after BSE intervention. Indeed, a study of eight healthy women who consumed the BSE containing 200 μmol of sulforaphane on average 50 min (27-75) prior to the start of reduction mammoplasty surgery reported that the ITC metabolite levels in breast tissue were 1.4 ± 3.2 and 2.0 ± 5.2 pmol/mg tissue for the right and the left breast, respectively, indicating large interindividual variations in bioavailability of ITCs in breast tissue [11]. Therefore, it is not surprising to see no strong correlations between the level of urinary ITC metabolites and the change of biomarkers in the BSE arm, as the urinary level of ITC metabolites does not necessarily reflect the ITC concentration in breast cancer tissue. Large interindividual variations, compounded by the small sample size of the study, may partly explain the nonsignificant findings for tissue biomarkers, despite the trend of changes in biomarkers aligned with biological functions of ITCs. Secondly, a randomized control trial design is critical. Urinary proteomic profiling revealed diverse expression patterns between the BSE and placebo arms even at the pre-intervention (Fig. 2A), which once again reflects the large interindividual variations at the molecular level at the baseline and underscores the importance of the placebo-controlled trial design. We observed a large number of urinary proteins altered in the study in both arms, and actually, the number of altered proteins between pre- and post-intervention was greater in the placebo arm than in the BSE arm (229 versus 178, Fig. 2B), underscore indicating the large placebo effect *per se*. By comparing to the placebo arm, we are able to focus pathway analysis on proteins altered specified by BSE intervention. The opposite direction of changes in tissue-level biomarkers between the BSE and placebo arms provides an additional support to ITC-specific effects on breast cancer.

In conclusion, this pilot intervention study was the first to demonstrate that anticancer activities of ITCs observed in *in vitro* and *in vivo* preclinical breast cancer models can be recapitulated in breast cancer patients, supporting the potential beneficial roles of ITC-containing cruciferous vegetables against breast cancer. Given that the multiple signaling pathways altered by ITC-rich BSE are involved in breast cancer growth and metastasis, the findings also provide evidence to support dietary recommendations on cruciferous vegetable intake among breast cancer survivors to improve breast cancer prognostic outcomes. However, a large, randomized intervention trial is needed to demonstrate long-term efficacy of ITCs against breast cancer, particularly long-term safety and efficacy of using ITC supplements among breast cancer survivors to improve prognosis. Urinary proteomic changes could serve as surrogate biomarkers to monitor the effect of ITCs in breast cancer patients in intervention trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BSE	broccoli sprout extract
ER	estrogen receptor
ITC	isothiocyanate
NQO1	NAD(P)H quinone dehydrogenase 1
TIL	tumor-infiltrating lymphocyte
IHC	immuno-histochemistry staining
ECOG	eastern cooperative oncology group
GERD	gastroesophageal reflux disease
FDR	false discovery rate
IPA	ingenuity pathway analysis
H&E	hematoxylin and eosin
LXR	liver X receptor
FXR	farnesoid X receptor
GST	glutathione S-transferase
NAT	N-acetyltransferase
SRF	serum response factor
IL-6	interleukin 6
MRTFB	myocardin related transcription factor B
S100A9	S100 calcium binding protein A9
LTF	lactotransferrin
SAA1	serum amyloid A1
ELANE	neutrophil elastase

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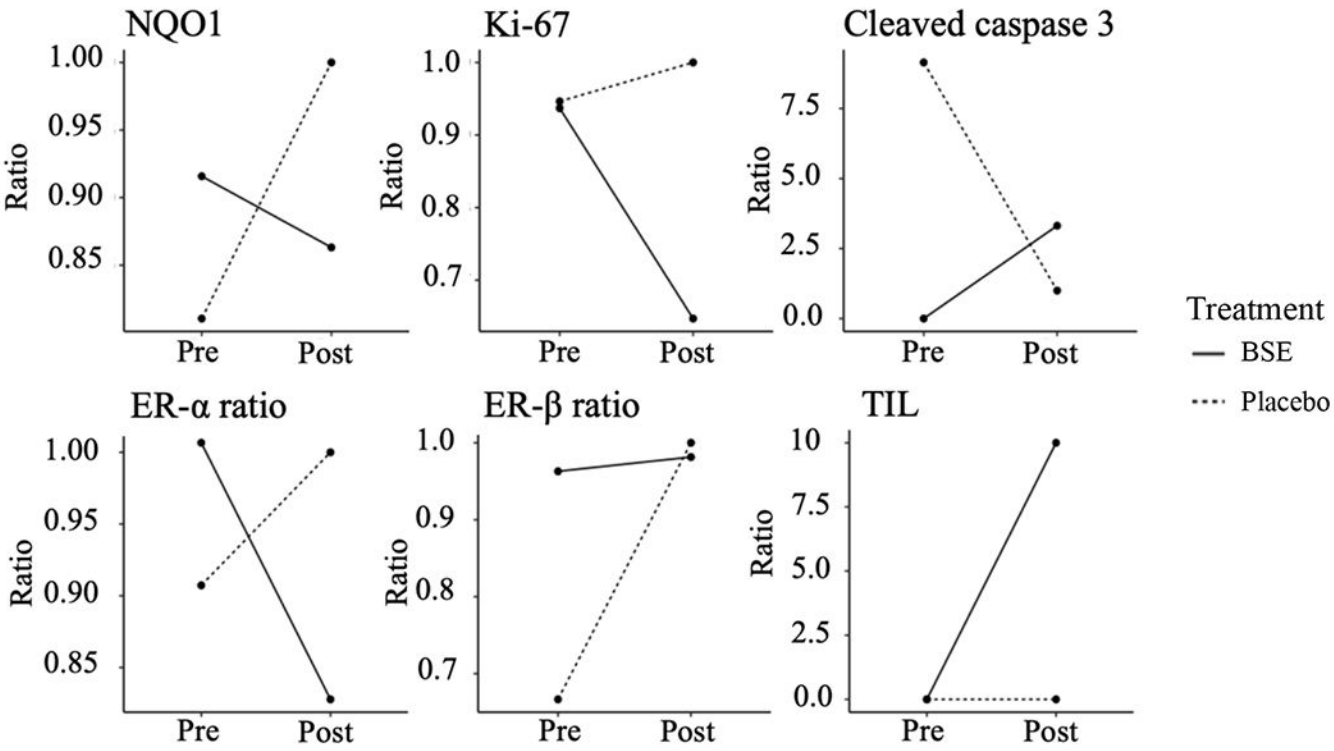

Figure 1.

Trend of changes in biomarker expressions between pre- and post-intervention in Broccoli Sprout Extract (BSE) trial. For each biomarker, the median of expression in post-intervention group of the placebo arm (Placebo post) was used as the reference and the ratios of the medians of expression in other three groups (BSE pre, BSE post, Placebo pre) to Placebo post were calculated and presented. NQO1: NAD(P)H quinone dehydrogenase 1, ER: estrogen receptor, TIL: tumor-infiltrating lymphocytes.

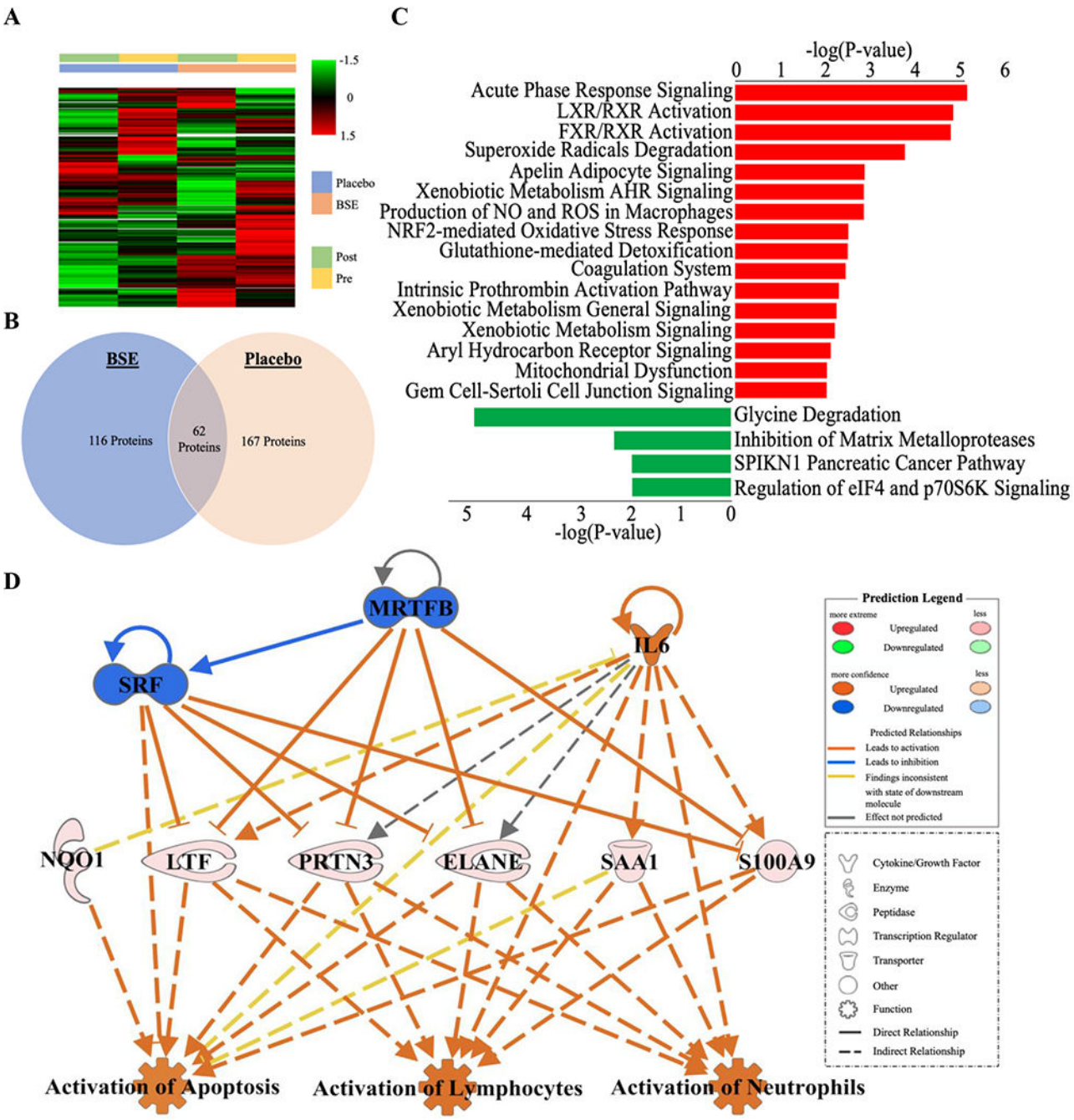

Figure 2.

Comparison of urinary proteomic profiles in pre- and post-intervention in Broccoli Sprout Extract (BSE) trial. 2A. Heatmap of urinary proteomic profiles; 2B. Significantly altered urinary proteins; 2C. Top enriched canonical pathways (-log(P-value) > 2) from upregulated (red) and downregulated (green) proteins in the BSE arm; 2D. Predicted effector network from proteins with top consistency scores in the BSE arm.

Table 1.

Demographic and clinical characteristics of breast cancer patients in Broccoli Sprout Extract (BSE) trial

	BSE (N=15)	Placebo (N=15)	Overall (N=30)	*P*-value[a]
Age at diagnosis, Mean ± SD	62.3±4.5	60.2±7.2	61.2±6.0	0.213
Race/Ethnicity, N (%)				
European American	14 (93.3)	15 (100)	29 (96.7)	0.309
African American	1 (6.7)	0	1 (3.3)	
Body mass index, N (%)				
Underweight & Normal (< 24.9)	3 (20.0)	3 (20.0)	6 (20.0)	0.915
Overweight (25.0 to 29.9)	4 (26.7)	5 (33.3)	9 (30.0)	
Obese (>29.9)	8 (53.3)	7 (46.7)	15 (50.0)	
Mean ± SD	31.9±7.7	29.7±6.1	30.8±6.8	0.471
ECOG scale, N (%)				
0	14 (93.3)	13 (86.7)	27 (90.0)	0.543
1	1 (6.7)	2 (13.3)	3 (10.0)	
Stage, N (%)				
DCIS	2 (13.3)	4 (26.7)	4 (13.3)	0.135
I	7 (46.7)	8 (53.3)	17 (56.7)	
II	5 (33.3)	3 (20.0)	8 (26.7)	
III	1 (6.7)	0	1 (3.3)	
Tumor grade, N (%)				
Well differentiated	2 (14.3)	4 (28.6)	6 (21.4)	0.315
Moderately differentiated	9 (64.3)	5 (35.7)	14 (50.0)	
Poorly differentiated	3 (21.4)	5 (35.7)	8 (28.6)	
Estrogen Receptor Status, N (%)				
Positive	13 (86.7)	14 (93.3)	27 (90.0)	0.543
Negative	2 (13.3)	1 (6.7)	3 (10.0)	
Progesterone Receptor Status, N (%)				
Positive	11 (73.3)	13 (86.7)	24 (80.0)	0.361
Negative	4 (26.7)	2 (13.3)	6 (20.0)	
HER2 Status, N (%)				
Positive	2 (13.3)	1 (6.7)	3 (10)	0.960
Negative/not determined	13 (86.7)	14 (93.3)	27 (90)	

[a]Fisher's exact test was used for categorical variables and student *t*-test was used for continuous variables.

Abbreviations: SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; DCIS, Ductal Carcinoma *in situ*; ER, Estrogen Receptor; PR, Progesterone Receptor; HER2, Human Epidermal Growth Factor Receptor 2.

Table 2.

Behavior characteristics of breast cancer patients in pre- and post-intervention in Broccoli Sprout Extract (BSE) trial

	BSE (N=15)			Placebo (N=15)				
	Pre N (%)	**Post N (%)**	***P*-value[a]**	**Pre N (%)**	**Post N (%)**	***P*-value[a]**	***P*-value[b]**	***P*-value[c]**
ITC-containing food intake [d]			< 0.05			0.22	0.43	N/A
No	9 (60.0)	15 (100)		12 (80.0)	15 (100)			
Yes	6 (40.0)	0 (0)		3 (20.0)	0 (0)			
Cigarette smoking			1.00			1.00	0.48	1.00
No	15 (100)	14 (93.3)		13 (86.7)	14 (93.3)			
Yes	0 (0)	1 (6.7)		2 (13.3)	1 (6.7)			
Alcohol drinking			0.60			0.70	1.00	0.08
No	12 (80.0)	14 (93.3)		11 (73.3)	9 (60.0)			
Yes	3 (20.0)	1 (6.7)		4 (26.7)	6 (40.0)			
Antibiotics use			1.00			N/A	1.00	N/A
No	14 (93.3)	15 (100)		15 (100)	15 (100)			
Yes	1 (6.7)	0 (0)		0 (0)	0 (0)			

[a] Comparison between pre- and post-intervention within each BSE and placebo arm using Fisher's exact test.

[b] Comparison between the BSE and placebo arms at pre-intervention using Fisher's exact test.

[c] Comparison between the BSE and placebo arms at post-intervention using Fisher's exact test.

[d] ITC-containing food includes broccoli, broccoli sprouts, cabbage, cauliflower, brussels sprouts, kale, collard greens, mustard greens, turnip greens, bok choy, water cress, radishes, turnips, kohlrabi, sauerkraut, horseradish, mustard and wasabi.

Table 3.

Compliance and Toxicity in Broccoli Sprout Extract (BSE) trial

	BSE (N=15)	Placebo (N=15)	Overall (N=30)	*P*-value[a]
Urinary ITC metabolites (μmol/g creatinine), Mean ± SD				
Pre-intervention	15.9±28.3	3.0±4.4	9.4±20.9	0.051
Post-intervention	228.0±152.0	1.3±1.7	114.6±156.3	<0.001
Net change, Range	6.6 - 598.7	−15.3 - 1.6	−15.6 - 598.7	<0.001
Maximal Grade of any adverse events, N (%)				
No	3 (20.0)	1 (6.7)	4 (13.3)	0.644
1	7 (46.7)	7 (46.7)	14 (46.7)	
2	2 (13.3)	4 (26.7)	6 (20.0)	
3	3 (20.0)	3 (20.0)	6 (20.0)	
Adverse events, N (%)				
Abdominal distension	4 (26.7)	1 (6.7)	5 (16.7)	
Diarrhea	1 (6.7)	0	1 (3.3)	
Dyspepsia	1 (6.7)	2 (13.3)	3 (10.0)	
Eructation	3 (20.0)	0	3 (10.0)	
Flatulence	4 (26.7)	1 (6.7)	5 (16.7)	
Nausea	4 (26.7)	1 (6.7)	5 (16.7)	
Abnormal thyroid blood test	2 (13.3)	3 (20.0)	5 (16.7)	
Neutrophil count decreased	1 (6.7)	1 (6.7)	2 (6.7)	
Arthralgia	1 (6.7)	0	1 (3.3)	
Dysgeusia	1 (6.7)	0	1 (3.3)	
Headache	2 (13.3)	1 (6.7)	3 (10.0)	
Hypertension	3 (20.0)	3 (20.0)	6 (20.0)	
Total events	27	13	40	

[a]Fisher's exact test was used for categorical variables and student *t*-test was used for continuous variables.

Abbreviations: ITC, isothiocyanate; SD, standard deviation.

Table 4.

Comparison of biomarkers in breast cancer tissue in Broccoli Sprout Extract (BSE) trial

	BSE (N=14)[a]			Placebo (N=15)			
	Pre	Post	*P*-value[b]	Pre	Post	*P*-value[b]	*P*-value[c]
NQO1, H-score	261 (125-283)	246 (163-285)	0.76	231 (173-292)	285 (179-291)	0.23	0.36
Ki67, H-score	9.1 (6.5-15.7)	6.3 (3.2-16.6)	0.22	9.2 (5.2-12.2)	9.7 (3.1-15.7)	0.89	0.21
ER-α ratio [d]	1.5 (0.9-2.6)	1.2 (0.6-2.0)	0.22	1.4 (1.2-2.1)	1.5 (1.0-2.1)	0.76	0.35
ER-α sum [e]	319 (240-356)	224 (151-329)	0.30	382 (236-446)	325 (203-490)	0.85	0.35
ER-β ratio [d]	0.5 (0.4-0.6)	0.5 (0.4-0.8)	0.18	0.4 (0.3-0.5)	0.5 (0.4-0.5)	0.09	0.86
ER-β sum [e]	147 (85-163)	147 (134-165)	0.54	132 (84-159)	151 (135-159)	0.17	0.68
Cleaved caspase 3 [f]	0 (0-3.2)	1.2 (0-10.8)	0.28	3.2 (0.2-20.3)	0.3 (0-8.9)	0.20	0.12
TILs [g]	0 (0-20)	10 (0-30)	0.16	0 (0-40)	0 (0-50)	1.00	0.16

[a] One participant was excluded due to missing biopsy tumor tissue specimen.

[b] Median and the interquartile range (IQR) were presented and compared between pre- and post-intervention using the Wilcoxon sign-rank test.

[c] Post-pre intervention changes were calculated and compared between treatment arms using an exact Wilcoxon rank-sum test.

[d] Ratio of nuclear to cytoplasm expression.

[e] Sum of nuclear and cytoplasm expression.

[f] Number of cleaved caspase 3 positive cells per mm^2.

[g] Percentage of tumor-infiltrating lymphocytes (TILs) in stroma excluding 3 DCIS cases in the BSE arm and 4 in the placebo arm.