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Urinary Isothiocyanate Levels, Brassica, and Human Breast Cancer[1]

Jay H. Fowke,[2] Fung-Lung Chung, Fan Jin, Dai Qi, Qiuyin Cai, Cliff Conaway, Jia-Rong Cheng, Xiao-Ou Shu, Yu-Tang Gao, and Wei Zheng

Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, Tennessee 37232-8300 [J. H. F., D. Q., Q. C., X-O. S., W. Z.]; American Health Foundation Cancer Center, Institute for Cancer Prevention, Valhalla, New York 10595 [F-L. C., C. C.]; and Shanghai Cancer Center, Shanghai, China 200032 [F. J., J-R. C., Y-T. G.]

ABSTRACT

Brassica **vegetable consumption (*e.g.,* Chinese cabbage) provides isothiocyanates (ITC) and other glucosinolate derivatives capable of inducing Phase II enzymes [*e.g.,* glutathione *S*-transferases (GSTM1, GSTT1, and GSTP1) and NADPH quinine oxidoreductase] and apoptosis, altering steroid hormone metabolism, regulating estrogen receptor response, and stabilizing cellular proliferation. Asian populations consuming large amounts of *Brassica* have a lower breast cancer incidence compared with Western populations; however, the association between *Brassica* consumption and breast cancer risk is uncertain. It is difficult to estimate glucosinolate exposure and degradation in humans, possibly limiting epidemiological investigations of *Brassica* and cancer associations. We conducted a case control investigation of breast cancer in Shanghai, China, using urinary ITC levels as a biological measure of glucosinolate intake and degradation in populations with habitual *Brassica* intake. A representative subgroup of 337 cases providing presurgery, fasting, and first-morning urine specimens was one-to-one matched (age, menopausal status, date of urine collection, and day of laboratory assay) to population controls. Urinary ITC levels were inversely associated with breast cancer [odds ratio (OR) $_{\text{Quartile 1}}$ = 1 (ref); OR_{Q2} = 0.9, 95% confidence interval (0.6, 1.4); OR_{Q3} = 0.7, (0.5, 1.1); OR_{Q4} = 0.5, (0.3, 0.8), adjusted for age, menopausal status, soy protein, fibroadenoma history, family breast cancer, physical activity, waist-to-hip ratio, body mass index, age at menarche, and parity in conditional logistic model]. This protective association persisted within post and premenopausal women. In contrast, total *Brassica* intake estimated from a food frequency questionnaire was not associated with breast cancer. Trends in the association between urinary ITC and breast cancer were more consistent with homozygous deletion of *GSTM1* or *GSTT1,* the *AA* genotype of *GSTP1* (A313G), or with the C allele of *NADPH quinine oxidoreductase* (C609T), although interactions were not statistically significant. In conclusion, greater *Brassica* vegetable consumption, as measured by the urinary ITC biomarker, was associated with significantly reduced breast cancer risk among Chinese women.**

INTRODUCTION

Breast cancer incidence is lower in Asian populations, suggesting that lifestyle practices and dietary habits affect breast cancer risk (1). In addition to greater soy and tea intake, and less fat intake, Asian populations habitually consume vegetables of the *Brassica* genus (*e.g.,* cabbage, bok choy, cauliflower, and turnip). These vegetables are a source of glucosinolates, an *N*-hydroxysulfate with a sulfur-linked β-glucose and a variable side chain containing either an alkyl, alkenyl, aromatic, indolyl, or perhaps other moiety (2). With chewing or cutting, the plant cell wall ruptures, and the enzyme myrosinase is released, cleaving the glucose from the glucosinolate. The gut microflora may have a level of myrosinase-like activity and the capacity to further degrade glucosinolates (3). The remaining aglucone intermediate is unstable and further degrades to yield a number of biologically active molecules, including sulforaphane and I3C.[3] These compounds are generally grouped and labeled as ITCs and indoles.

In animal models of breast cancer, tumor growth is inhibited by *Brassica* consumption, or ITC or I3C administration (4–8). The ITCs are both inducers of, and substrates for, Phase II enzymes, including GSTs and NQO1 (9–11). These detoxifying enzymes may protect cells against cancer initiation by neutralizing endogenous and exogenous electrophiles in breast tissue. Furthermore, the ITCs from *Brassica* have been found to induce apoptosis (10, 12–16). The persistence, distribution, and excretion of ITCs may depend on GST activity (17–21), and genetic polymorphisms in *GSTM1, GSTT1, GSTP1,* or perhaps other Phase II enzymes, such as *NQO1*, may limit the ability of ITCs to stabilize cellular proliferation and induce apoptosis in breast tissues. The indoles also induce apoptosis and regulate cellular proliferation (22–26). In breast tumor and other cells, indoles down-regulate expression of the estrogen-responsive pS2 and cathepsin-D genes and induce p21 expression consistent with inhibition of proliferation and G_1-S stasis (27–30). Recent preliminary investigations in PC-3 prostate cancer cells suggest that indoles down-regulate the epidermal growth factor receptor and reduce activity of the anti-apoptotic proto-oncogene *AKT* (31). Furthermore, indole exposure may shift estrogen metabolism to favor the catechol estrogens with less affinity for the estrogen receptor (32) and exhibit tamoxifen-like properties (29, 33).

However, in humans, the relationship between *Brassica* consumption and breast cancer risk is uncertain. Investigations have found null associations (34–37), protective but not statistically significant associations (38–40), and statistically significant protective associations (41). In one sense, these studies are consistent, because *Brassica* has not been associated with increased breast cancer risk. However, there may be several explanations for the inability to distinguish a "null" from "protective" association. Across these studies, population *Brassica* consumption may be below a biologically effective level, the measured dietary intake may not be during an etiologically relevant time period, or the analyses may be confounded. Furthermore, FFQs may not measure the sources of variability in ITC and indole exposure, including cultivar, the consumption of less common vegetables, vegetable preparation methods, or storage conditions (42–46).

To address dietary assessment limitations, a biomarker of dietary *Brassica* has been developed to compliment existing FFQ assessment methods common in epidemiological investigations. The ITCs from *Brassica* are excreted in urine as dithiocarbamates (47) and may be measured by HPLC after deconjugation and a cyclocondensation reaction with 1,2-benzenedithiol (47, 48). This urinary ITC marker provides a measure of total ITC exposure for each subject and has been positively correlated with habitual *Brassica* intake in an Asian study population (19). Previously, this urinary ITC marker was successful in identifying a protective interaction between *Brassica,* Phase II enzymes, and lung cancer in Chinese men (49). In this study, we

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[2] To whom requests for reprints should be addressed, at 6110 MCE, Vanderbilt University Medical Center, Nashville, TN 37232-8300. Phone: (615) 936-2903; E-mail: jay.fowke@vanderbilt.edu.

[3] The abbreviations used are: I3C, indole-3-carbinol; ITC, isothiocyante; GST, glutathione *S*-transferase; HPLC, high-performance liquid chromatography; FFQ, food frequency questionnaire; WHR, waist-to-hip ratio; SBCS, Shanghai Breast Cancer Study; NQO1, NADPH quinine oxidoreductase; BMI, body mass index; CV, coefficient of variation; PEITC, phenethyl isothiocyante; OR, odds ratio; CI, confidence interval.

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investigated the association between breast cancer, urinary ITC levels, and the interactions with GSTM1, GSTT1, GSTP1, and NQO1 genotypes among women living in Shanghai, China.

MATERIALS AND METHODS

Participants. Details of the SBCS, recruitment protocols, and data collection protocols have been reported previously (50, 51). Briefly, the SBCS is a population-based case control study conducted among Chinese in urban Shanghai, China. Eligible breast cancer patients were diagnosed during the period August 1996 to March 1998, between 25 and 64 years of age, and permanent residents of Shanghai, China. Cases were identified through our rapid case ascertainment system and supplemented by the population-based Shanghai Cancer Registry. There were 1601 eligible breast cancer cases identified for the study, and interviews were completed from 1459 (91.1%) of eligible cases. The major reasons for nonparticipation were refusal (n = 109, 6.8%), death before interview (n = 17, 1.1%), and inability to locate (n = 17, 1.1%). Two senior pathologists confirmed each cancer diagnosis through the review of tumor slides.

Community controls were randomly selected from the female general population using the Shanghai Resident Registry, a registry of all adult residents in urban Shanghai. Only women who lived at the listed address during the study period were eligible. Controls were frequency matched by 5-year age categories using projections of the age distribution of breast cancer patients, and in-person interviews were completed on 90.3% of the 1724 eligible controls (refusals: n = 166, 9.6%; death or previous cancer diagnosis: n = 2, 0.1%). Informed consent was obtained from all participants.

A urine sample was collected from 98.7% of cases and 99.8% of controls. After urine collection, 125 mg of ascorbic acid were added to ~100 ml of urine to prevent oxidation of labile compounds, and samples were immediately transported on ice (0°C-4°C) to the central laboratory for processing and long-term storage at −70°C within 6 h of collection.

Data Collection. A structured questionnaire was used to obtain information on demographics, reproductive history, hormone use, dietary habits, disease history, physical activity, tobacco and alcohol use, weight history, and family history of cancer for each participant. Weight, height, and the circumferences of the waist and hips were measured by trained staff. Habitual dietary intake regarding the past 5 years was measured by a validated FFQ specifically designed to measure intake of foods commonly consumed in Shanghai, China. Nutrient scores were computed using the Chinese Food Composition Table (52). Previous analysis of the SBCS found positive association with age, education, family history, menopausal age, age at first live birth, WHR, and BMI and inverse associations with age at menarche and exercise (50).

Study Design. We conducted an individually matched case control study within the SBCS to increase the overall comparability of cases and controls in studying quantitative biomarkers. Preliminary sample size calculations suggested that 350 case control pairs would provide reasonable statistical power. Cases in the ITC substudy were SBCS cases providing a fasting, first-morning urine specimen collected before any cancer treatment or surgery. For each case, a control was selected from the pool of controls completing the study, individually matched to cases by age (±3 year), menopausal status, and date of sample collection and interview (±30 days). Successful matches were completed for 350 case control pairs for urinary isothiocyanate analysis. Thirteen cases were found not to meet study inclusion criteria after we conducted the urinary ITC assays; thus, our analysis included 337 case control pairs. Table 1 compares these 337 cases and 337 controls to cases and controls in the SBCS across established breast cancer risk factors. By chance, ITC substudy cases were 1 year older at menarche, and 6% more controls exercised. Overall, across almost all demographic and reproductive parameters, the ITC substudy participants were generally comparable with the SBCS population.

Analysis of Urinary Isothiocyanates. The method for analysis of urinary total ITCs and their thiol metabolites described in detail (48) was slightly revised. Briefly, frozen urine samples were thawed and vortexed; 1-ml samples were placed in 2-ml glass vials and centrifuged (2800 rpm) for 15 min to sediment suspended matter, then placed on ice. Triplicate aliquots of 100 μl of clarified urine were carefully pipetted into 2 ml of HPLC vials (Chromacol, Inc., Trumbull, CT) containing 600 μl of a degassed 2-propanol solution of 10 mM 1,2-benzenedithiol (Lancaster Synthesis, Inc., Waldham, NM) and 500 μl of degassed 0.1 M potassium phosphate (pH 8.5). The reaction mixtures in capped vials were vortexed and incubated for 2 h at 65°C in a shaking water bath. The reaction mixtures were cooled and centrifuged (2800 rpm, 20 min) before analysis of the reaction product 1,3-benzenditthiol-2-thione by HPLC.

After incubation, samples were analyzed by reverse-phase HPLC using a Waters μBondapak C_{18} (150 × 3.9 mm) with a Waters C_{18} guard column and detection wavelength of 365 nm. The mobile phase consisted of a mixture of methanol and H_2O (7:3 volume for volume) with a flow rate of 1.75 ml/min. A Shimadzu model SCL-10A controller, dual LEC-10AS pumps, and SIL-10A autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD) were used; Axxiom 727 software (Axxiom Chromatography, Inc., Moorpark, CA) was used to collect and integrate HPLC data. Concurrent triplicate standards of the *N*-acetyl conjugate of PEITC-*N*AC were prepared in 20 mM phosphate buffer (pH 5.0) at concentrations of 0 (H_2O), 5, 15, and 25 μM; concurrent

Table 1 *Comparison of SBCS participants and ITC substudy participants for selected breast cancer risk factors*

SBCS: n = 1459 cases, n = 1556 controls; ITC substudy: n = 337 cases, n = 337 controls. Subjects with missing values were excluded from the analysis. Wilcoxon's rank-sum test or Fisher's exact test (two tailed) used for continuous or categorical comparisons, respectively. χ^2 test used for education.

	Cases			Controls		
A.	ITC median (IQR)[a]	SBCS median (IQR)	P	ITC median (IQR)	SBCS median (IQR)	P
BMI	23.2 (4.0)	23.2 (4.3)	0.90	22.5 (4.3)	22.8 (4.4)	0.29
WHR	0.81 (0.07)	0.81 (0.07)	0.49	0.80 (0.07)	0.80 (0.07)	0.45
Age (years)	47 (12)	47 (11)	0.81	46 (12)	46 (14)	0.35
Age at menarche (years)	15 (3)	14 (3)	0.03	15 (3)	15 (3)	0.31
Age at first live birth (years)[b]	27 (5)	27 (5)	0.97	26 (4)	26 (4)	0.88
Age of menopause (years)[c]	49 (6)	49 (5)	0.72	48 (5)	48 (5)	0.79
Number of children	1 (1)	1 (1)	0.77	1 (1)	1 (1)	0.55

	Cases					Controls				
B.	n	%	n	%	P	n	%	n	%	P
Exercise regularly (no)	71	21%	273	19%	0.32	104	31%	392	25%	0.03
Premenopausal	219	65%	990	64%	0.95	216	64%	990	64%	0.87
Breast cancer in first-degree relative	11	3%	54	4%	0.87	6	2%	38	2%	0.55
Ever had breast fibroadenoma	27	8%	140	9%	0.41	15	4%	78	5%	0.67
Current tobacco user	4	1%	10	1%	0.31	7	2%	36	2%	0.99
Education										
No formal education	16	5%	37	3%	0.67	15	5%	67	5%	0.80
Elementary school	28	8%	96	9%		29	9%	102	9%	
Middle + high school	248	74%	836	75%		259	77%	915	75%	
Professional, college, and above	45	13%	153	13%		31	9%	138	11%	

[a] IQR, interquartile range.
[b] Among women with any live births.
[c] Among postmenopausal women only.

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Table 2 *Comparison of breast cancer risk factors between cases and matched controls* (*n* = 337 pairs)

		Cases		Controls			
		n	%	*n*	%	OR	95% CI
BMI	≥30	15	4%	10	3%	1.6	0.7, 3.6
WHR	≥0.85	72	21%	50	15%	1.6	1.1, 2.4
Leisure activity	No activity	71	21%	104	31%	1.8	1.2, 2.6
Age at menarche	<11 yrs	5	1%	1	<1%	5.0	0.6, 43
Age at menopause[a]	≥54 yrs	13	19%	11	18%	1.3	0.3, 5.9
Age at first live birth[b]	>30 yrs	73	22%	52	18%	1.5	1.0, 2.2
Number of children	≤1	231	69%	219	65%	1.5	0.9, 2.4
Family breast cancer	Yes	11	3%	6	2%	1.8	0.7, 4.9
Fibroadenoma history	Yes	27	8%	15	4%	1.9	1.0, 3.6
Current smoker	Yes	4	1%	7	2%	0.6	0.2, 2.0
GSTM1[c]	Null (−)	172	53%	161	53%	1.0	0.7, 1.3
GSTT1	Null (−)	151	46%	113	38%	1.4	1.0, 1.9
GSTP1	GA/GG	116	36%	87	29%	1.5	1.0, 2.0
NQO1	TT	65	21%	38	13%	1.4	0.9, 2.3

[a] Postmenopausal women only, <48 yrs of age at menopause as referent.
[b] Age at first birth >30 years or no live births.
[c] Frequencies may be <337 case control pairs because of missing genotype data.

standards were analyzed with each batch of urine samples. PEITC-*N*AC was prepared, and purity and structure were verified by nuclear magnetic resonance and HPLC in the Organic Synthesis Laboratory (53). A standard curve [1–100 μM PEITC-*N*AC in 20 mM phosphate buffer (pH 5.0), in triplicate], prepared and analyzed weekly, was used for quantification of urinary total ITC concentrations.

All laboratory analyses were blind to the case control status. Each case and matched control were handled as a pair and analyzed on the same day to reduce variability. The interbatch CV [(CV = SD/mean) × 100] was 3.4%. The intrabatch CV across five ITC standards (2, 5, 10, 15, and 100 μM) was 9.64, 6.64, 5.57, 5.11, and 3.84%, respectively.

Determination of Urinary Creatinine. Urine samples (100 μl) were diluted to 2 ml with deionized H_2O, briefly vortexed, then pipetted into a 500-μl sample cup for analysis using a Vitros 500 Clinical Chemistry Analyzer (Johnson & Johnson Clinical Diagnostics, Rochester, NY). High- and low-level human urine creatinine controls were concurrently analyzed to insure that the instrument was performing satisfactorily.

Genotyping Methods. Genomic DNA was extracted from buffy coat fractions using the Puregene DNA isolation Kit (Gentra Systems, Minneapolis, MN) following the manufacturer's protocol. DNA concentration was measured by PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). Five to 10 ng of genomic DNA were used for each PCR. The laboratory staff was blind to the identity of the subject. Quality control samples (water, CEPH 1347-02 DNA, as well as blinded and unblinded DNA samples) were included in genotyping assays.

The *NQO1* C609T genetic polymorphism, reported to have lower activity (54), was evaluated by the PCR-RFLP method. The primers for the PCR reaction were: F: 5′-TCC TCA GAG TGG CAT TCT GC-3′ and R: 5′-TCT CCT CAT CCT GTA CCT CT-3′. Each PCR product was subjected to *Hin*fI digestion. The C→T substitution at nucleotide 609 creates a *Hin*fI restriction site. The PCR product (230 bp) with C allele was digested to two fragments (195 and 35 bp), whereas the PCR product with T allele was digested to three fragments (151, 44, and 35 bp). A multiplex PCR protocol was used to analyze simultaneously for the presence or absence of *GSTM1* and *GSTT1* genes (55). The *Albumin* gene was used as an internal control. Although these assays did not distinguish between heterozygote- and homozygote-positive genotypes, they conclusively identify the null genotypes. The *GSTP1* A313G polymorphism Ile105Val is located within the substrate-binding site of *GSTP1,* and substitution for the G allele is believed to have differential affinity for electrophilic compounds (56). The interaction between this GSTP1 polymorphism and colorectal cancer has been described recently; thus, our analysis will extend *GSTP1* function across Asian study populations and cancer sites (57). This polymorphism (58) was determined by PCR-RFLP method following the method reported previously (59).

Statistical Analysis. Urinary ITC concentrations were standardized to creatinine levels (micromoles of ITC/milliliters of urine/milligrams of creatinine) to adjust for variability in urine volume. A paired *t* test was used to compare cases and controls across continuously scaled measurements, and conditional logistic regression (ORs and 95% CIs) was used to compare matched cases and controls across categorically scaled breast cancer risk factors. The nonparametric Wilcoxon sign rank test compared urinary ITC levels or Brassica intake across case control pairs while avoiding evaluation of log-transformed data. The Wilcoxon rank-sum test was used to compare urinary ITC levels across Phase II enzyme genotypes, whereas the Kruskal-Wallis test was used to compare ITC levels by stage of breast cancer.

Quartile categories of urinary ITC were determined using the control series distribution. ORs and 95% CIs were calculated using multivariable conditional logistic regression (SAS, version 8.2). Results were almost identical using an unconditional logistic model, with inclusion of matching covariates, and results from the conditional modeling approach are reported. Covariates included as potential confounders include those reproductive, behavioral, genetic, and body size measures found to be associated with breast cancer risk (Table 2). Gene–ITC interactions were evaluated by inserting the corresponding cross-product terms into the model. Statistical significance of the interaction terms was evaluated using the log-likelihood test; however, the multiplicative interaction may be conservative, and thus, we also describe overall patterns of associations between ITC levels and breast cancer across genotypes. Tests for trend between increasing categories of urinary ITC levels and breast cancer were determined by the significance of a continuous variable representing each participant's ITC category inserted into the logistic model.

RESULTS

In this individually matched case control investigation, premenopausal subjects (64%) averaged 43 years of age (range: 28–56 years), whereas postmenopausal subjects (36%) averaged 56 years of age (range: 40–64 years). Cases had a higher BMI and waist-to-hip ratio and lower activity levels (Table 2). Patterns in reproductive indexes were consistent with greater lifetime estrogen exposure among cases. Few subjects used tobacco [cases: $n = 4$ (1%); controls: $n = 7$ (2%)], and only three cases and seven controls worked in an agricultural or rubber/plastics industry. Homozygous deletion of *GSTT1,* the AA genotype of GSTP1, or the TT genotype of *NQO1* were marginally associated with breast cancer. Cases and controls had similar energy, total fat, and soy protein intakes (data not shown).

Cases reported nonsignificantly less habitual *Brassica* intake (cases: median = 77.4 grams/day; controls: median = 81.5 grams/day; $P = 0.16$). Consistently, cases had significantly lower urinary ITC levels compared with controls (Table 3). Case control differences in urinary ITC levels were slightly larger with deletion of *GSTM1* or *GSTT1* or with the G or C alleles of *GSTP1* and *NQO1,* respectively. Within either the cases or controls, urinary ITC levels were fairly stable across categories of each genotype. Urinary ITC levels differed the most with homozygous deletion of *GSTT1* among controls; however, this difference was not statistically significant ($P = 0.22$).

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Table 3 *Urinary ITC levels (μmol/ml/mg creatinine) by Phase II enzyme genotype*

		Cases		Controls		
		n	Median	*n*	Median	*P*[a]
ITC		337	1.71	337	2.31	<0.01
Gene[b]						
GSTM1	+	152	1.73	141	2.31	0.24
	−	172	1.71	161	2.50	<0.01
GSTT1	+	171	1.77	187	2.31	0.15
	−	151	1.65	113	3.00	0.01
GSTP1	AA	208	1.76	217	2.31	0.06
	AG/GG	116	1.64	87	2.42	0.04
NQO1	CC/CT	251	1.71	249	2.31	0.02
	TT	65	1.68	38	2.07	0.55

[a] *P* from Wilcoxon's sign rank test for paired ITC data or rank-sum test for ITC by genotype analysis.
[b] Genotype frequencies may be <337 case control pairs because of missing genotype data.

Urinary ITC levels did not vary with stage of breast cancer diagnosis [median ITC levels: stage 0/1: 1.7 (n = 82), stage 2A/B: 1.7 (n = 209), stage 3/4: 1.8 (n = 35), and stage unknown: 1.7 (n = 12); P = 0.62].

Habitual *Brassica* intake estimated by FFQ was not consistently associated with breast cancer (Table 4), although there was a marginally significant protective trend among postmenopausal women attributable to the highest level of self-reported *Brassica* intake and breast cancer. In contrast, participants categorized to the highest quartile of urinary ITC excretion were 50% less likely to be diagnosed with breast cancer [OR = 0.5, 95% CI (0.3, 0.8); Table 4], with a consistent trend across lower ITC categories. Removal of subjects currently using tobacco or working in agriculture, rubber, or plastics had no effect on these results. Furthermore, adjustment for *GSTM1, GSTT1, GSTP1,* or *NQO1* genotypes did not substantively alter the results. This protective association persisted within pre and postmenopausal women. Further adjustment for Phase II enzyme genotypes did not effect the observed associations among premenopausal women, although trends among the smaller group of postmenopausal women were less stable [OR_{Q1} = 1 (ref), OR_{Q2} = 0.6 (0.2, 1.6), OR_{Q3} = 0.9 (0.3, 2.8), and OR_{Q4} = 0.5, (0.2, 1.7), adjusted for Phase II genotypes and other covariates].

To investigate the effects of Phase II enzyme activity, the association between ITC and breast cancer was determined for each geno-

Table 4 *Breast cancer and urinary ITC excretion or Brassica intake*

Conditional logistic regression, with categorization of ITC or *Brassica* set at quartiles of the control distributions.

	Cases	Controls	OR[a]	95% CI	OR[b]	95% CI
ITC			All subjects			
Q1	104	87	1.0		1.0	
Q2	97	87	0.9	(0.6, 1.3)	0.9	(0.6, 1.4)
Q3	84	87	0.7	(0.4, 1.1)	0.7	(0.5, 1.1)
Q4	56	87	0.5	(0.3, 0.8)	0.5	(0.3, 0.8)
			$P_{trend} < 0.01$		$P_{trend} < 0.01$	
			Postmenopausal subjects			
Q1	36	35	1.0		1.0	
Q2	35	36	0.7	(0.3, 1.4)	0.7	(0.3, 1.7)
Q3	29	28	0.7	(0.3, 1.7)	0.7	(0.3, 1.9)
Q4	22	29	0.5	(0.2, 1.3)	0.6	(0.2, 1.7)
			$P_{trend} = 0.20$		$P_{trend} = 0.38$	
			Premenopausal subjects			
Q1	68	52	1.0		1.0	
Q2	62	51	0.9	(0.5, 1.6)	0.9	(0.5, 1.7)
Q3	55	59	0.7	(0.4, 1.3)	0.7	(0.4, 1.2)
Q4	34	58	0.4	(0.2, 0.8)	0.5	(0.2, 0.9)
			$P_{trend} = 0.01$		$P_{trend} = 0.01$	
Brassica			All subjects			
Q1	90	88	1.0		1.0	
Q2	85	87	0.9	(0.6, 1.4)	0.9	(0.6, 1.5)
Q3	96	86	1.1	(0.7, 1.7)	1.2	(0.8, 1.9)
Q4	70	87	0.7	(0.5, 1.2)	0.8	(0.5, 1.3)
			$P_{trend} = 0.36$		$P_{trend} = 0.79$	
			Postmenopausal subjects			
Q1	31	26	1.0		1.0	
Q2	34	30	1.0	(0.4, 2.4)	1.2	(0.4, 3.2)
Q3	32	32	0.7	(0.3, 1.8)	1.2	(0.4, 3.3)
Q4	25	40	0.4	(0.2, 1.0)	0.4	(0.1, 1.1)
			$P_{trend} = 0.02$		$P_{trend} = 0.07$	
			Premenopausal subjects			
Q1	59	62	1.0		1.0	
Q2	51	57	0.8	(0.5, 1.4)	0.8	(0.4, 1.4)
Q3	64	54	1.2	(0.7, 2.0)	1.2	(0.7, 2.0)
Q4	45	47	0.9	(0.5, 1.7)	1.0	(0.5, 1.8)
			$P_{trend} = 0.78$		$P_{trend} = 0.79$	

[a] Crude ORs.
[b] Adjusted ORs for soy protein, fibroadenoma, family breast cancer, leisure activity, WHR, BMI, age at menarche, and number of children.

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Table 5 *Urinary ITC levels and breast cancer, by Phase II genotypes*

All ORs and 95% CI adjusted for soy protein, age, menopausal status, fibroadenoma history, leisure activity, WHR, BMI, and number of children. P_{trend}, trend in ORs across ITC categories, within each genotype. P_{int}, log likelihood test for significance of interaction terms for urinary ITC categories and genotype.

Gene		Urinary ITC categories Q1	Q2	Q3	Q4	P_{trend}
GSTM1	+	1.0	0.9 (0.5, 1.8)	0.7 (0.3, 1.5)	0.6 (0.3, 1.4)	0.20
	−	1.0	1.0 (0.5, 1.9)	0.9 (0.4, 1.7)	0.5 (0.2, 0.9)	0.05
					P_{int} = 0.82	
GSTT1	+	1.0	1.1 (0.6, 1.9)	0.8 (0.4, 1.5)	0.6 (0.3, 1.3)	0.20
	−	1.0	0.7 (0.3, 1.4)	0.9 (0.4, 2.1)	0.4 (0.2, 0.9)	0.03
					P_{int} = 0.44	
GSTP1	AA	1.0	0.8 (0.5, 1.5)	0.7 (0.4, 1.4)	0.6 (0.4, 1.3)	0.18
	AG/GG	1.0	1.0 (0.4, 2.7)	1.0 (0.4, 2.5)	0.5 (0.2, 1.2)	0.12
					P_{int} = 0.98	
NQO1	CC/CT	1.0	0.9 (0.5, 1.5)	0.8 (0.5, 1.6)	0.6 (0.3, 1.1)	0.09
	TT	1.0	1.7 (0.5, 5.9)	1.9 (0.6, 6.7)	0.5 (0.1, 1.8)	0.51
					P_{int} = 0.43	

type (Table 5). No statistically significant multiplicative interaction was observed. Trends appeared to be stronger or more consistent within the GSTM1-null, GSTT1-null, GSTP1-AA, and NQO1 C allele genotypes, and higher urinary ITC levels were necessary to observe a protective association among subjects with the G allele of *GSTP1* or TT genotype of *NQO1*. The protective trend with the C allele of *NQO* was statistically significant among premenopausal women (P_{trend} = 0.04).

DISCUSSION

Laboratory research suggests that *Brassica* consumption reduces breast cancer risk, perhaps through induction of detoxifying Phase II enzymes (60), interaction with estrogen metabolism or the estrogen signaling pathway (29, 30, 61, 62), induction of apoptosis (10, 22–26), and modified expression of cell cycle regulators (27, 63). However, full-scale epidemiological studies have been challenged to develop an exposure index for *Brassica* ITCs and indoles. We investigated the association between breast cancer and urinary ITC levels, a biomarker of overall *Brassica* vegetable consumption and an estimate of exposure to at least one phytochemical group of interest. In our study, greater urinary ITC excretion was associated with lower pre and postmenopausal breast cancer.

Breast carcinogenesis requires years, and a biomarker-breast cancer association requires inference from a single biomarker measurement to a habitual dietary pattern. Although ITCs are excreted within 1–3 days after a single *Brassica* meal (64), this biomarker may provide an index of habitual *Brassica* intake because *Brassica* consumption levels are high and consumed with great frequency in China. Groups with steady-state *Brassica* consumption would have a steady-state ITC excretion reflecting typical glucosinolate exposure for that group. Seow *et al.* (19) found habitual *Brassica* intake, averaging ~40 grams/day, was favorably associated with urinary ITC levels among Chinese living in Singapore. In our investigation, participants reported consuming an average of 92 grams/day *Brassica* during the previous 5 years, and almost all urine specimens had detectable ITC level (3% nondetects; 12 cases and 8 controls). We also have found that habitual *Brassica* intake estimated from our FFQ significantly increased with urinary ITC levels (65), reflecting a traditional diet with strong links to regional agriculture. We could not rule out a possible contribution of side-stream smoke exposure to urinary ITC levels; however, few participants used tobacco, and the insensitivity of the ITC assay to tobacco smoke thiols would further reduce the effects of any tobacco smoke exposure (66). Urine collection protocols were standardized across cases and controls, with the collection of first-morning, fasting, urine specimens, and minimizing case control differences caused by recent intake.

One of the strengths of the urinary ITC dietary biomarker is that it provides an estimate of *Brassica* consumption independent of recall bias or other potential FFQ reporting errors (67–69). Most FFQs are not designed to measure a narrowly defined food group such as *Brassica*. Less frequently consumed vegetables, although potentially potent, may not be on the food list, and it is not possible to have portion-size guides for each food item. Furthermore, urinary ITC levels provide a measure of the internalized exposure to *Brassica* ITCs and perhaps other *Brassica* phytochemicals, accounting for variability in glucosinolate levels across species of *Brassica,* myrosinase-like activity, cooking methods, storage, plant size and age, and weather and soil conditions (42, 70). Our observation of a consistent protective association between urinary ITC levels and breast cancer was in contrast to the weaker associations observed with total *Brassica* intake, suggesting that the urinary ITC biomarker may indeed be a better measure of habitual Brassica intake in an Asian population.

Phase II enzyme activity may affect breast cancer risk (71, 72) or ITC excretion (19). However, we found no evidence of confounding because of variability in Phase II genotypes between cases and controls. Any associations between Phase II enzyme genotypes and breast cancer were weak, and in contrast to Seow *et al.* (19), urinary ITC levels were somewhat higher among *GSTT1*-null subjects. No statistically significant interactions were identified, but there were several patterns to consider. Protective trends were more consistent with the *GSTM1 null, GSTT1 null, GSTP1-AA,* and *NQO1-C* genotypes. Phase II enzyme function may contribute to the biological response to *Brassica* by affecting the transport of phytochemicals to target tissues and the neutralization of electrophilic species (21). Additionally, ITCs transported to breast tissue may induce breast *NQO1* expression (73) to neutralize transitory semiquinones from 2- and 4-hydroxy catechol (74–76). Speculation aside, the nonsignificant patterns observed will require confirmation.

Study results using indole or ITC in animal models of chemoprevention have been mixed. Tumor status was dependent on the ITC or indole dose, congener profile, time of administration, animal model, and tumor-inducing agent (77–81). In humans, short-term I3C administration did not produce adverse effects (82, 83), although the long-term effects on health remain unknown. In contrast, *Brassica* consumption is nontoxic, inexpensive, and provides a complex exposure to glucosinolates and nutrients, possibly providing protection against many common cancers (84–86). Greater overall fruit and vegetable consumption may not be sufficient to reduce breast cancer risk (39). However, there is accumulating evidence that *Brassica* vegetables hold potential in breast cancer prevention. We found that urinary ITC levels, a glucosinolate biomarker in Asian populations, were

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significantly associated with reduced breast cancer risk in pre and postmenopausal women.

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