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Green tea intake, *MTHFR/TYMS* genotype and breast cancer risk: the Singapore Chinese Health Study

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The tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) has been reported to act as a cancer preventive agent through folate pathway inhibition in experimental studies. We hypothesized that if folate pathway inhibition is the mechanism of cancer preventive activities of EGCG, then the protective effect against breast cancer would be stronger among women with low dietary folate intake and the high-activity methylenetetrahydrofolate reductase (*MTHFR*) and thymidylate synthase (*TYMS*) genotypes. In a nested case–control study of 380 women with incident breast cancer and 662 controls within the Singapore Chinese Health Study, we found no association between either green tea intake or gene polymorphisms of *MTHFR* (*C677T* and *A1298C*) and *TYMS* (*1494 ins/del*) and breast cancer risk. However, among women with low folate intake (<133.4 μg/day), weekly/daily green tea intake was inversely associated with breast cancer risk compared with less green tea intake [odds ratio (OR) = 0.45, 95% confidence interval (CI) = 0.26–0.79, *P* for interaction = 0.02]. Among women with high folate intake (≥133.4 μg/day), green tea intake was not associated with breast cancer. Similarly, among women possessing the high-activity *MTHFR/TYMS* genotypes (0–1 variant allele), weekly/daily versus less frequent green tea intake was associated with lower breast cancer risk (OR = 0.66, 95% CI = 0.45–0.98), which was observed even more strongly among those who also had low folate intake (OR = 0.44, 95% CI = 0.22–0.89) than high folate intake (OR = 0.92, 95% CI = 0.55–1.54). This association was not observed among women possessing the low-activity genotypes (2–4 variant alleles). Our findings suggest that folate pathway inhibition may be one mechanism through which green tea protects against breast cancer in humans.

Introduction

Tea, brewed from dried leaves of the plant *Camellia sinensis*, is one of the most widely consumed beverages in the world. Although black tea is the most commonly consumed tea in Western countries, green tea is favored in Asian countries, especially in Japan and China. Green tea differs from other types of tea in the abundant presence of tea polyphenols, known as catechins and its major compound, (−)-epigallocatechin-3-gallate (EGCG), which have been reported to act as a cancer chemoprotective agent. Although the exact mechanism by which green tea polyphenols may protect against cancers in human is not completely understood, there have been extensive *in vitro* and animal studies of possible mechanisms (1–4). One suggested mechanism is the inhibition of dihydrofolate reductase by EGCG, which stimulates apoptosis and cell cycle arrest of cancer cells. In cell culture and animal models, EGCG has been shown to inhibit cell growth in a variety of cancers including skin, prostate, breast, lung and colon (5).

To date, the few epidemiologic studies have shown inconsistent results regarding the association between green tea intake and risk of breast cancer (6–12). Given inconsistent findings of epidemiologic studies, we were interested in evaluating whether genetic variation modifies the relationship between green tea intake and risk of breast cancer. In a case–control study with Asian-American women, green tea catechin protected against breast cancer among carriers of low-activity catechol-*O*-methyltransferase alleles but not among those who possessed high-activity catechol-*O*-methyltransferase alleles (13). We reported previously a low risk of breast cancer among women with higher green tea intake and the low-activity genotype of angiotensin-converting enzyme gene among Singapore Chinese women (14). In the present study, we were specifically interested in determining whether the association between green tea intake and risk of breast cancer differs among women in terms of genetic variation of the folate pathway enzymes: methylenetetrahydrofolate reductase (MTHFR) and thymidylate synthase (TYMS).

MTHFR catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, whereas TYMS uses the 5,10-methylenetetrahydrofolate as a methyl group donor for conversion of uracil to thymidine for DNA synthesis. Two common polymorphisms, *C677T* and *A1298C*, in the coding region of the *MTHFR* gene are associated with reduced enzyme activity of MTHFR *in vitro* (15–18). Compared with the wild-type *677 CC* genotype, the activity level of MTHFR is ∼30 and 60% in the homozygous variant *677 TT* and the heterozygous variant *677 CT* genotypes, respectively (17). The *A1298C* polymorphism also decreases MTHFR activities especially accompanied with the *C677T* polymorphism (19). Similarly, one potentially functional polymorphism, a 6 bp deletion in the *TYMS* 3′-untranslated region, has been shown to decrease *TYMS* expression (20,21). We hypothesized that if methylation inhibition in the folate pathway is the mechanism for cancer preventive effects of EGCG, then the preventive effect of green tea would be stronger among women with high-activity MTHFR and TYMS compared with women with low activity of these enzymes. We also hypothesized that the effect of EGCG on breast cancer risk would be modified by dietary folate intake. In other words, women with fewer variant alleles in *MTHFR* and *TYMS* genes who drink green tea regularly with less folate intake would have the lowest risk of breast cancer.

Abbreviations: CI, confidence interval; EGCG, (−)-epigallocatechin-3-gallate; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; SCHS, Singapore Chinese Health Study; TYMS, thymidylate synthase.

Subjects and methods

Study subjects

The subjects were selected from participants of the Singapore Chinese Health Study (SCHS), a population-based prospective cohort investigation to elucidate the role of diet and its interaction with genetic factors in risk of cancer. The study design and subject recruitment of the SCHS have been described elsewhere (22). Briefly, 63 257 Chinese women and men aged 45–74 years belonging to the Hokkien or Cantonese dialect group in Singapore were enrolled in the study between April 1993 and December 1998. At recruitment, information on lifestyle factors, usual diet and reproductive history was obtained through in-person interviews. The dietary component of the questionnaire was validated through a series of 24 h food recalls (22). Each subject was asked to estimate his or her usual intake frequencies and portion sizes for 165 food and beverage items during the past 12 months. The frequency of green tea intake was defined as nine categories: never or hardly ever, one to three times a month, once a week, two to three times a week, four to six times a week, once a day, two to three times a day, four to five times a day and six or more times a day. This study was approved by the Institutional Review Boards at the University of Minnesota, the University of Southern California and the National University of Singapore.

Between April 1994 and July 1999, we attempted to collect blood and single-void urine specimens from a random 3% sample of study enrollees (Figure 1). Details of the biospecimen collection, processing and storage procedures have been described previously (23). If the subject refused to donate blood, buccal cell samples were requested and collected if the subject consented. Out of 1059 female cohort participants contacted for biospecimen donation, blood (n = 514) or buccal cells (n = 164) were collected from 678 subjects, representing a participation rate of 64%.

The control group for the present study was comprised of this subcohort of women who were free of a history of breast cancer as of 31 July 2007. Six hundred and sixty-two subjects satisfied this criterion (4 had positive history at enrollment, 2 had missing genotype and 10 developed breast cancer during the follow-up). In January 2000, eligibility of the biospecimen subcohort was extended to all surviving cohort enrollees.

Post-diagnostic biospecimen samples were requested from all incident cases of female breast cancer starting from April 1994. Incident cases of breast cancer were identified through linkage with the population-based cancer registry in Singapore. As of 31 July 2007, 736 cases of incident breast cancer had been identified among the female cohort subjects via this linkage. Histological and staging information on all breast cancer diagnoses were confirmed by manual review of the pathology reports and clinical charts. The number of incident breast cancer cases within the cohort is comparable with the expected number of cases based on age- and sex-specific incidence rates of breast cancer for all Chinese in Singapore (24,25). Genotype information was determined for 380 (51.6%) incident cases of breast cancer with blood or buccal cell samples (124 pre-diagnostic and 256 post-diagnostic biospecimens). Compared with women with breast cancer who did not participate due to lack of biospecimens or genotype information, the study participants who were enrolled as cases were more likely to be of Hokkien (54.2 versus 44.5%, $P < 0.01$) and attained regular period at an earlier age (55.8 versus 43.4% at 14 years old or earlier, $P = 0.02$). The two breast cancer groups were otherwise similar.

Genotyping methods

For the genotype determination, blood and buccal cell samples were shipped on dry ice to the University of Southern California. Genomic DNA was purified from buffy coats of peripheral blood and buccal cell specimens using a PureGene Blood Kit (Gentra System, Minneapolis, MN) or a QIAamp 96 DNA Blood Kit (Qiagen, Valencia, CA). Genotyping assays were developed for the *MTHFR C677T* (rs1801133), *MTHFR A1298C* (rs1801131) and the *TYMS3′*-untranslated region polymorphism (*1494 ins/del6*) (rs16430). Genotype frequencies were determined using TaqMan assays as reported previously (26).

Statistical analysis

The *t*-tests (for continuous variables) or chi-square tests (for categorical variables) were used to compare the cases and controls in baseline characteristics and risk factors for breast cancer. Data were analyzed by standard methods for unmatched case–control studies (27). Unconditional logistic regression models were used to examine the associations between green tea intake frequency and risk of breast cancer and the possible modifying effect of *MTHFR*/*TYMS* gene polymorphism and folate intake on the tea–breast cancer association. The associations were estimated by odds ratios (ORs), 95% confidence intervals (CIs) and *P* values. The *P* values for trend were calculated based on the logistic regression model with ordinal value for the exposure variables.

Relevant demographic factors including age (continuous, years) at recruitment, year of recruitment (1993–1998), dialect group (Cantonese and Hokkien), level of education (no formal education, primary school, secondary school or higher), black tea intake (continuous, number of cups per month) and established risk factors for breast cancer including body mass index (continuous, kilogram per square meter), age when period became regular (<12, 13–14, 15–16, 17+ years or period never became regular) and number of live births (none, 1–2, 3–4 and 5+) were adjusted for in the analysis (23,28). Statistical analysis was carried out using the SAS software Version 9.1 (SAS Institute, Cary, NC). All reported *P* values were calculated by two sided and $P < 0.05$ was considered statistically significant.

Results

Table I shows the distributions of baseline demographic characteristics and potential risk factors between case and control subjects. Cases and controls were comparable with respect to age at enrollment, dialect group, body mass index and current smoking status. Similar to previous reports from the SCHS (14,23), the cases were more educated, attained regular period at an earlier age, had fewer numbers of live births and reached menopause later than the controls. As use of hormone replacement and family history of breast cancer are well-established risk factors of breast cancer, the proportion of hormone replacement use and family history of breast cancer were both slightly higher among cases than controls in the present study population, as expected. However, the differences did not reach statistical significance. It is probably because that breast cancer incidence and the rate of hormone replacement use are relatively low in Singapore. None of the green tea, black tea and coffee intake frequencies were different between cases and controls. The frequency of green tea intake only

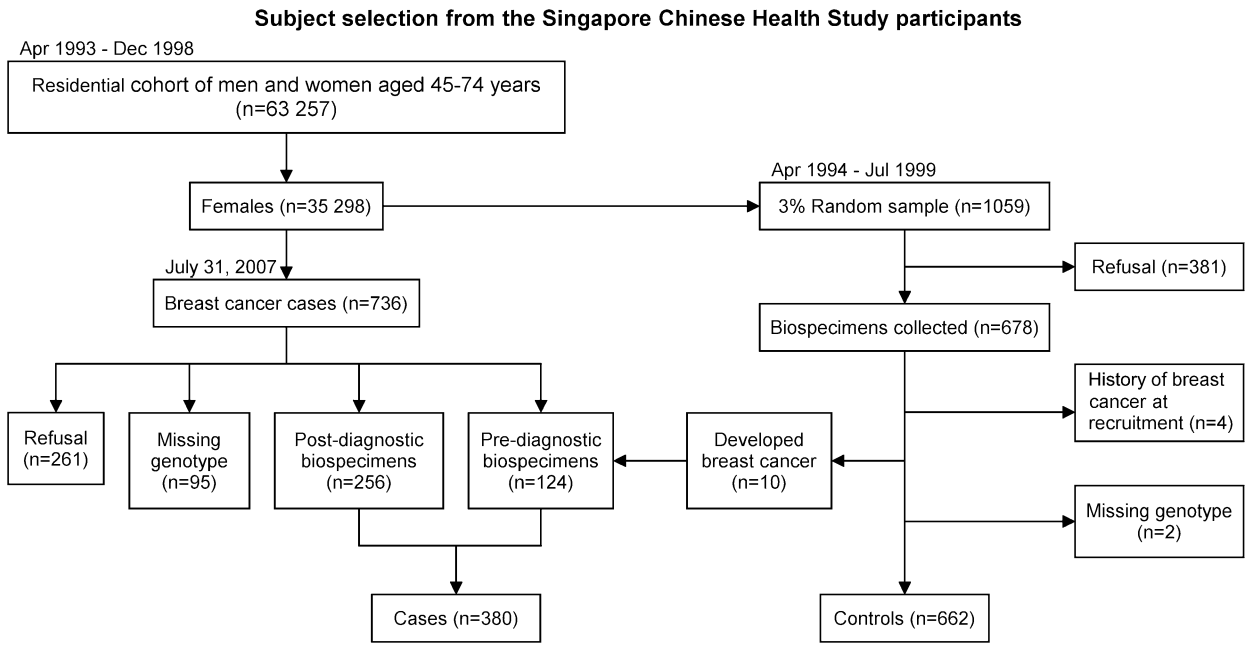

Fig. 1. Subject selection from the SCHS cohort.

Table I. Demographic characteristics and risk factors in breast cancer cases and control subjects

	Cases (%), n = 380	Controls (%), n = 662	P
Age (mean ± SD)	55.8 ± 7.6	55.8 ± 8.0	0.99
Dialect group			
Cantonese	55.5	50.0	0.09
Hokkien	44.5	50.0	
Highest level of education			
No formal education	30.8	38.5	0.04
Primary school	41.1	37.5	
Secondary school or higher	28.1	24.0	
Body mass index (kg/m^2)			
<20	11.1	14.2	0.19
20 to <24	56.3	55.9	
24 to <28	22.9	23.3	
28+	9.7	6.6	
Age at period became regular			
<13	14.7	13.6	0.05
13–14	41.0	36.0	
15–16	30.3	29.9	
17+/never became regular	14.0	20.5	
Number of live births			
0	12.1	7.2	<0.01
1–2	35.5	28.0	
3–4	34.2	39.3	
5+	18.2	25.5	
Age at menopause[a]			
<50	28.7	36.1	<0.01
50–54	53.0	54.8	
55+	18.3	9.1	
Use of hormone replacement			
Never	92.4	94.3	0.45
Former users	1.3	1.2	
Current users	6.3	4.5	
Family history of breast cancer[b]			
No	97.9	98.8	0.26
Yes	2.1	1.2	
Cigarette smoking			
Never smokers	93.4	93.7	0.88
Ever smokers	6.6	6.3	
Black tea intake			
None or <weekly	76.8	79.6	0.30
≥Weekly	23.2	20.4	
Coffee intake			
<Daily	28.7	28.0	0.80
Daily	71.3	72.0	
Dietary folate intake[c] (μg/day)			
<133.4	45.3	48.3	0.34
≥133.4	54.7	51.7	

[a]Including only subjects who were 55 years or older at recruitment (202 cases and 332 controls).
[b]Family history of breast cancer among first-degree relatives.
[c]Median dietary folate intake for all female participants in the SCHS = 133 μg/day.

was not associated with the risk of breast cancer with or without adjustment for covariates (Table II).

Controls were in Hardy–Weinberg equilibrium for both *MTHFR C677T* and *A1298C* (P = 0.16 and 0.48, respectively). The *MTHFR C677T* and *A1298C* sites were in strong linkage disequilibrium (r^2 = 0.10, P < 0.01) among controls, as others have reported previously (29). These findings suggest a founder effect in which each alteration evolved on separate wild-type alleles. On the other hand, *TYMS 1494 ins/del* and *MTHFR C677T* or *A1298C* were not in linkage disequilibrium. Neither the *MTHFR 677* variant genotypes that have high/low (*CT*) or low/low (*TT*) activity alleles nor the *MTHFR1298* variant genotypes with high/low (*AC*) or low/low (*CC*) activity alleles were associated with the risk of breast cancer (Table II). Similarly, the *TYMS* gene polymorphism, i.e. *6bp ins/del*, was not associated with

Table II. Green tea intake, *MTHFR/TYMS* genotype and risk of breast cancer

	Cases (n = 380)	Controls (n = 662)	Adjusted OR[a] (95% CI)	P
Green tea intake				
None or <weekly	279	467	1.00	0.41
Weekly to <daily	50	119	0.65 (0.45–0.94)	
Daily	51	76	1.00 (0.82–1.22)	
MTHFR677 genotypes				
CC	239	393	1.00	0.24
CT	120	226	0.87 (0.66–1.15)	
TT	21	43	0.79 (0.45–1.38)	
MTHFR1298 genotypes				
AA	225	387	1.00	0.54
AC	139	234	1.02 (0.77–1.34)	
CC	16	41	0.70 (0.38–1.30)	
Sum of *MTHFR* variant alleles				
0	119	182	1.00	0.27
1	226	416	0.83 (0.62–1.11)	
2	35	64	0.84 (0.70–1.36)	
TYMS genotypes				
−6 bp/−6 bp	193	328	1.00	0.54
−6 bp/+6 bp	157	273	0.98 (0.75–1.29)	
+6 bp/+6 bp	30	61	0.83 (0.51–1.35)	
Sum of *MTHFR* and *TYMS* variant alleles				
0–1	224	366	1.00	0.25
2–4	156	296	0.86 (0.66–1.12)	

[a]Adjusted for age, year of enrollment, education, dialect, body mass index, age when period became regular, number of live births and black tea intake.

the risk of breast cancer. The total number of variant alleles in *MTHFR* and *TYMS* genes was not associated with the risk of breast cancer. However, risk of breast cancer was statistically significantly lower among women who drank green tea weekly or more compared with non-green tea drinkers among women with 0 or 1 variant allele in *MTHFR* and *TYMS* genes after adjustment (OR = 0.66, 95% CI = 0.45–0.98). There were no associations between green tea intake and risk of breast cancer for any single genotype of *MTHFR* and *TYMS* (Table III).

Since our hypothesized mechanism for cancer preventive activity of EGCG involves folate metabolism, we considered that dietary folate intake might modify the association between green tea intake and risk of breast cancer. Overall, the average daily dietary folate intake among the study population was 149.2 μg/day (range = 31.2–590.2 μg/day), which is significantly lower than the recommended daily allowance both in Singapore (200 μg/day) and the USA (400 μg/day). Dietary folate intake was not statistically significantly different between cases and controls (Table I). However, among all female participants in the SCHS, dietary folate intake was ∼30% higher among women who drank green tea weekly or daily (mean ± SD = 174.6 ± 75.2 μg/day) than non-drinkers (135.2 ± 62.7 μg/day). All subjects were categorized as high (≥133.4 μg/day) or low (<133.4 μg/day) folate consumers with a cut point at the median folate intake for all female participants in the SCHS. Among women whose dietary folate intake was low, risk of breast cancer was statistically significantly lower in women who drank green tea weekly or more (OR = 0.45, 95% CI = 0.26–0.79) than those with less frequent green tea intake (Table IV). This association was not observed among women with high folate intake. The inverse association between green tea intake and risk of breast cancer among women with high-activity alleles was even stronger among those who also had low dietary folate intake (OR = 0.44, 95% CI = 0.22–0.89, P for interaction = 0.02) (Table IV). This inverse association between green tea intake and risk of breast cancer was not observed among women with high folate intake.

Table III. Green tea intake and risk of breast cancer by *MTHFR/TYMS* genotype

	Green tea intake				Adjusted OR[a] (95% CI)
	None or <weekly		≥Weekly		
	Cases	Controls	Cases	Controls	
MTHFR677 genotypes					
CC	177	272	62	121	0.70 (0.48–1.03)
CT	86	158	34	68	0.79 (0.47–1.33)
TT	16	37	5	6	5.03 (0.64–39.47)
MTHFR1298 genotypes					
AA	170	286	55	101	0.86 (0.58–1.28)
AC	96	155	43	79	0.83 (0.51–1.36)
CC	13	26	3	15	0.39 (0.07–2.23)
TYMS genotypes					
−6 bp/−6 bp	144	227	49	101	0.72 (0.47–1.08)
−6 bp/+6 bp	112	193	45	80	0.89 (0.56–1.40)
+6 bp/+6 bp	23	47	7	14	0.49 (0.12–1.90)
Number of variant alleles[b]					
0–1	172	256	52	110	0.66 (0.45–0.98)
2–4	107	211	49	85	0.96 (0.61–1.50)

[a]OR for ≥weekly green tea intake group with none or <weekly green tea intake group as a reference group; adjusted for age, year of enrollment, education, dialect, body mass index, age when period became regular, number of live births and black tea intake.
[b]Total number of variant alleles in *MTHFR677*, *MTHFR1298* and *TYMS*.

Table IV. Green tea intake, combined *MTHFR/TYMS* genotype and risk of breast cancer by folate intake

Green tea intake	Low folate intake[a]			High folate intake[a]		
	Cases	Controls	Adjusted OR[b] (95% CI)	Cases	Controls	Adjusted OR[b] (95% CI)
None or <weekly	152	252	1.00	127	215	1.00
≥Weekly	20	68	0.45 (0.26–0.79)	81	127	1.04 (0.72–1.50)
0–1 variant alleles						
None or <weekly	100	134	1.00	72	122	1.00
≥Weekly	13	39	0.44 (0.22–0.89)	39	71	0.92 (0.55–1.54)
2–4 variant alleles						
None or <weekly	52	118	1.00	55	93	1.00
≥Weekly	7	29	0.50 (0.19–1.30)	42	56	1.18 (0.67–2.07)

[a]Low and high folate intake categories use the cutoff point at 133.4 μg/day (median of all female participants in the cohort).
[b]Adjusted for age, year of enrollment, education, dialect, body mass index, age when period became regular, number of live births and black tea intake.

Discussion

The present study suggests a possible protective effect of green tea against breast cancer among women with high-activity genotypes of the *MTHFR* and *TYMS* genes. This effect was even stronger among those who were low consumers of dietary folate. To our knowledge, this is the first epidemiologic study that examines a gene–environment interaction between *MTHFR* and *TYMS* gene polymorphisms and green tea intake on its potential preventive activity against breast cancer development. The findings of the present study support the hypothesis that green tea polyphenols may influence the folate pathway and that genetic polymorphisms of enzymes in the folate pathway may modify the tea–breast cancer association.

EGCG inhibits dihydrofolate reductase, which catalyzes the conversion of dihydrofolate to tetrahydrofolate in the folate pathway. The folate pathway is vital for nucleotide synthesis, especially for rapidly replicating cells such as cancer cells. In fact, cancer chemopreventive agents such as antifolates induce apoptosis and thus affect the steady cell population. The mechanism of anticarcinogenic activity of EGCG was recently found to be similar to antifolate chemotherapeutic agents *in vivo* (30).

Epidemiologic studies have not shown consistent results regarding the association between tea intake and breast cancer risk. Majority of studies in the USA or Europe, where black tea is preferred to green tea, have reported no association between tea intake and breast cancer risk, probably because of the extremely small amount of tea polyphenol intake (31–34). Although a few studies have evaluated the association in an Asian population, where green tea consumption is relatively high, study findings have again been inconsistent (6–8,14). One possible explanation is that gene–nutrient interaction may modify the anticarcinogenic activity of green tea polyphenols in humans. Numerous studies have investigated the effect of *MTHFR* gene polymorphisms on risk of breast cancer; however, results have been discordant (35–46). In the present study, the risk of breast cancer was not associated with *MTHFR* and *TYMS* genotypes.

Considering *MTHFR* as a regulating enzyme in the folate pathway, the other possible explanation for inconsistency among previous study results is the nutrient–nutrient interaction between folate and tea polyphenols. We found a lower risk of breast cancer associated with regular green tea intake only among women with low folate intake. Furthermore, the inverse association between risk of breast cancer and regular green tea intake was the strongest among women possessing high-activity *MTHFR* and *TYMS* genotypes whose dietary folate intake was low. Our results correspond with a limited number of previous studies that examined the interaction among folate intake, genotype and breast cancer risk (47–49). Folate depletion increases the sensitivity of cancer cell lines to EGCG *in vitro* (30). Thus, EGCG does not have to compete with folate and can effectively inhibit

dihydrofolate reductase. Findings from the present study lend support to the hypothesis that green tea polyphenols may have anticarcinogenic properties through competing with folate in the folate pathway.

The present study has several strengths. Singapore is a small city-state where all citizens have good access to specialized medical care. The nation-wide cancer registry has been available since 1968 and has been shown to be comprehensive in its recording of cancer cases (24). Thus, the ascertainment of incidence cases of breast cancer can be assumed to be comprehensive. Secondly, our study population is Asian with a relatively high frequency of green tea intake compared with the USA or European study cohorts. Consequently, we were able to collect a relatively large number of green tea drinkers for cases and controls. Thirdly, green tea intake was assessed prior to breast cancer diagnosis and therefore can be presumed to be free of recall bias. Furthermore, average folate intake among the current study population was significantly lower compared with the average daily dietary folate intake for adult women in the USA (300–350 μg/day) (50), which offered the opportunity to evaluate antifolate effects of EGCG in relative absence of opposition from folate.

The major limitation of the present study is lack of detailed data on green tea intake. Although green tea drinking is more popular in Singapore compared with Western countries, Singapore, having been a major port for foreign trade, has developed the unique mixture of Asian and Western cultures, which has also had impacts on beverage drinking habits. As shown in Table II, the number of daily green tea drinkers was relatively small in the present study population. Therefore, daily green tea drinkers were combined into the category of weekly or more frequently drinkers in the final analyses. Our findings require confirmation in other large cohorts with high exposures to green tea. Moreover, the EGCG content in green tea varies depending on how it is prepared; in the current study, information on the methods used to brew the tea (infusion time and strength) was unavailable. In addition, we assessed tea intake only at a single time point (baseline). However, non-differential misclassification of exposure status tends to minimize the underlying relative risk toward the null. It is unlikely that such exposure misclassification leads to a spurious association between exposure and disease risk. Next, biospecimens for genotype determination were donated by only 64% of controls and 51.6% of cases. However, refusal to donate biospecimens occurred before genotypes were determined; therefore, it is unlikely that genotypes could affect subjects' decisions to donate their biospecimens. Thus, it is unlikely that refusal to donate biospecimens led to selection bias regarding genotype. Lastly, our result that dietary folate intake was significantly higher among women with breast cancer who drank green tea more frequently suggests that green tea drinking habits may be associated with a healthier lifestyle. If so, there might be some unmeasured factors that is reflected by green tea intake and decreased breast cancer risk.

In summary, the present study provides supportive epidemiologic evidence to the previously suggested hypothesis that EGCG stimulates apoptosis and cell cycle arrest through inhibition of the folate cycle. Genetic polymorphisms in *MTHFR* and *TYMS* were found to modify the preventive activities of green tea polyphenols against breast cancer. Our findings were demonstrated in a population with low average folate intake. These results need to be confirmed by future studies with large sample sizes, considering folate intake as a potential effect modifier in the interaction among tea polyphenols, *MTHFR*/*TYMS* genotypes and breast cancer risk.

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Conflict of Interest Statement: None declared.

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