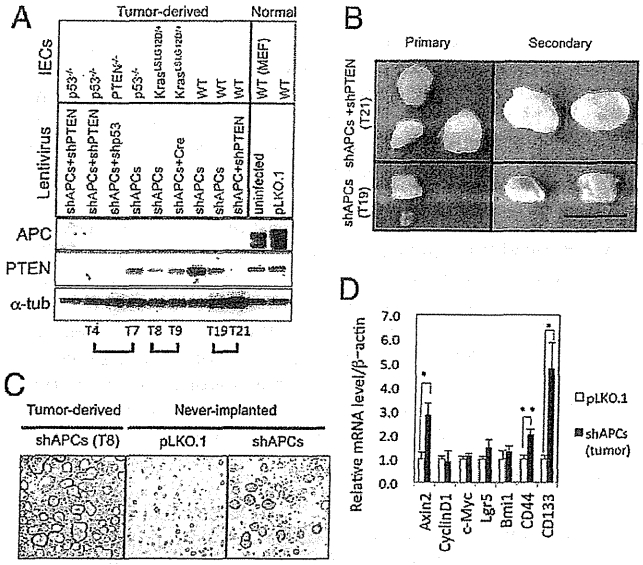

Fig. 6. Induction of cancer stem–like properties in tumors through *APC* suppression. (*A*) *APC* knockdown in tumors. Immunoblotting analysis of tumor-derived organoids for APC and PTEN. Mouse embryonic fibroblast (MEF) and normal IECs are positive controls. T4/7, T8/9, and T19/21 derived from identical primary cells, respectively. (*B*) Serial transplantation of tumors. s.c. tumors T19 (shAPCs) and T21 (shAPCs+shPTEN) in primary sites (*Left*) and corresponding secondary tumors after retransplantation (*Right*). (Scale bar, 10 mm.) (*C*) Sphere-forming assay. Representative images after 2-wk suspension culture are shown. (*D*) qPCR analysis for Wnt target genes and stem cell marker genes. Mean ± SD ($n = 3$) is shown; $^*P < 0.01$; $^{**}P < 0.05$.

engineered mouse, independently of intestinal microenvironment, and solely with primary IECs, providing an alternative way to model CRC in vitro. There were several issues to be resolved in the course of developing the model. First was to achieve stable transduction of stem cells, instead of gene targeting in embryonic stem (ES) cells. Despite initial technical difficulties, we eventually established a lentivirus-based transduction method, which was as simple as a routine subculture but highly efficient in obtaining stably transduced organoids. This methodological advantage was in sharp contrast to retroviral transduction of only cycling cells, with more complicated procedures but much less efficiency, definitely requiring drug selection to obtain stably transduced organoids (43). Both high infection efficiency and the growth advantage of inactivating tumor suppressors helped us conduct experiments without drug selection, which eliminated its potential side effects and enabled us to use vectors without selection markers or puro-resistant IECs from the *Kras*$^{LSLG12D/+}$ mouse (34). We further showed that Cre-mediated recombination of a floxed allele could be achieved in organoids. Thus, overexpression and knockdown could be simply achieved for many genes in WT IECs and gene disruption in conditionally gene-targeted IECs.

The second was in vitro expansion of *APC*-inactivated organoids. Inactivation of *APC* in the intestine is normally established by a stochastic second hit in heterozygous mice or by complete loss in mice homozygous for a floxed allele in a spatiotemporally regulated manner (22, 44, 45). Although rounded cystic organonids were available by 3D culture of *APC*-deficient adenoma developed in earlier studies (26, 27), *APC* loss and subsequent tumor development have been exclusively achieved in vivo, to which inflammation or interactions within the microenvironment might have played critical roles (17, 18). In some settings, tumor development itself could not be achieved due to organ failure induced immediately after *APC* loss (28). Thus, it was initially unclear whether we could achieve *APC* inactivation in organoids and its subsequent propagation thoroughly in vitro. IECs transduced with shAPC frequently failed to propagate, which might be partially in line with adverse effects caused by acute *APC* loss (28). We incidentally noted that this could be overcome by cointroducing all of the shAPC clones. A possible explanation could be that pooled shAPC clones yielded variations in the magnitude of Wnt activation, thereby increasing the probability of achieving the "just-right" signaling (46). Alternatively, cooperation among off-target effects by pooled clones could have contributed. Although the underlying mechanism remains to be investigated, organoids that grew out indeed carried shAPCs and phenocopied those derived from *APC*-deficient adenoma. Based on the high similarity, we reasoned that propagated organoids with shAPCs might likely be an in vitro equivalent to adenoma. We then took advantage of this situation for further analysis.

The third was the strict definition of tumor in this model, which was definitely required to relate the results to earlier studies in vivo. We tentatively defined tumors as nodules replacing coinjected Matrigel with proliferating epithelial glands at 6 wk postinjection. Only if tumors proved lethal at an earlier point were nodules exceptionally diagnosed on death. Accordingly, nonlethal nodules at 2 wk after injection were not treated as tumors. Acquisition of the potential for sphere formation and serial transplantation and induction of CSC markers were confirmed in tumor-derived organoids, clearly indicating that they had indeed undergone transformation. These results tend to support the validity of our definition of tumors.

With this experimental system, the relevance of known genetic alterations in CRC could be essentially recapitulated, either individually or in the context of *APC* loss, as in *PTEN* loss (11, 12) and *Kras* activation (13–15, 36, 37). With regard to *p53* loss, its genetic cooperation with *APC* loss was negative in the heterogeneous genetic background (31, 47) but proved to be positive in a congenic background (9, 10), consistent with this study. These findings highlight the relevance of conducting the analysis on genetic interaction in exactly the same genetic background as achieved in our model, which otherwise requires multiple backcrossing. Thus, validation of candidate genes or genetic cooperation will be warranted, leading to quick identification of the genes to be prioritized for further investigations from many candidates (19, 20) before, or even without, generation of gene-modified mice. Also, generation of tumors with defined genotypes could be facilitated. The custom-made "genetically clean" cell lines would become valuable resources for identification of effective compounds or therapeutic targets by high-throughput screening or in preclinical studies.

Considering similarities in outcome and approach, our in vitro model might well be comparable to those two types of in vivo studies, in which *APC* was subject to acute deletion in the intestine, rather than studies with the *APC*-heterozygous mutant mouse (15). One is *APC* loss in an Lgr5$^+$ stem cell–specific manner, which quickly gives rise to adenoma (22, 26, 48). The other is *APC* loss either focally or entirely in the intestine. Local injection of adenovirus-Cre induced adenoma in the distal colon, although transduction was achieved in only a limited area (44, 45). Cre-mediated inducible and acute loss of APC throughout the intestine led to morbidity within 5 d (28), but the crypts that were rescued by harvesting 2 d after APC loss proved tumorigenic in nude mice (49). In both cases, *APC* inactivation was achieved in gene-modified mice in vivo, presumably by cooperating with the microenvironment. Besides, special conditions such as cell type–specific gene ablation or crypt harvest at specific times were necessary. In contrast, in our model, *APC* inactivation was simply achieved in WT IECs in vitro, without any other type of cells or experimental conditions, obviously facilitating intestinal tumorigenesis studies. As organoid culture is optimized for Lgr5$^+$ stem cells (24), it is conceivable that Lgr5$^+$ stem cells were predominantly transformed in our model. On the other hand,

lentiviral transduction could also target quiescent stem cells, which could be marked by Bmi1 (42), Lrig1 (50), mTert (51), or HopX (52), in a mutually overlapping but distinct manner. Moreover, even Lgr5$^-$ differentiated cells could dedifferentiate to reacquire stem cell properties and initiate tumorigenesis (49). Thus, there is a possibility that tumor initiation could take place through many different pathways, including reprogramming of nonstem cells. In this regard, our model might provide unique opportunities in addressing this issue in an unbiased way, as the entire process of tumorigenesis could be simply recapitulated without predefined conditions on tumor-initiating cells.

In conclusion, we developed a unique in vitro model for CRC, with which genetic interactions in both tumor initiation and progression will be simply but genuinely analyzed. By serving as an alternative or complement to the standard approaches, it would likely accelerate CRC research.

Materials and Methods

Singly dissociated intestinal cells were lentivirally transduced in vitro. Organoids were maintained for 4 wk in Matrigel and injected into nude mice to evaluate tumorigenicity. Several weeks after the implantation, the tumors were subjected to histological analysis or 3D culture to obtain a pure population of tumor-derived organoids, which were further analyzed by Western blotting, qPCR, and sphere-forming assay. Extended materials and methods are available in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank the Core Facility Division and Central Animal Division in the National Cancer Center Research Institute for technical support in histological and animal studies, respectively. We thank Elaine Fuchs for providing LV-Cre pLKO.1, Hiroshi Ohshima for *p53*-mutant mice, and Akira Suzuki for *PTEN* floxed mice. We also thank Ibuki Kobayashi and Sachiko Dobashi for technical assistance. This study was supported by Grants-in-Aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan and a grant from the Japan Chemical Industry Association Long Range Research Initiative. K. Onuma was a recipient of the Research Resident Fellowship from the Foundation for Promotion of Cancer Research (Japan).

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Iwasaki et al. Breast Cancer Research 2010, 12:R88
http://breast-cancer-research.com/content/12/5/R88

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Green tea drinking and subsequent risk of breast cancer in a population to based cohort of Japanese women

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Abstract

Introduction: Although many *in vitro* and animal studies have demonstrated a protective effect of green tea against breast cancer, findings from epidemiological studies have been inconsistent, and whether high green tea intake reduces the risk of breast cancer remains unclear.

Methods: In this Japan Public Health Center-based Prospective Study, 581 cases of breast cancer were newly diagnosed in 53,793 women during 13.6 years' follow-up from the baseline survey in 1990 to 1994. After the five-year follow-up survey in 1995 to 1998, 350 cases were newly diagnosed in 43,639 women during 9.5 years' follow-up. The baseline questionnaire assessed the frequency of total green tea drinking while the five-year follow-up questionnaire assessed that of two types of green tea, *Sencha* and *Bancha/Genmaicha*, separately.

Results: Compared with women who drank less than one cup of green tea per week, the adjusted hazard ratio (HR) for women who drank five or more cups per day was 1.12 (95% confidence interval (CI) 0.81 to 1.56; *P* for trend = 0.60) in the baseline data. Similarly, compared with women who drank less than one cup of *Sencha* or *Bancha/Genmaicha* per week, adjusted HRs for women who drank 10 or more cups per day were 1.02 (95% CI 0.55 to 1.89; *P* for trend = 0.48) for *Sencha* and 0.86 (0.34 to 2.17; *P* for trend = 0.66) for *Bancha/Genmaicha*. No inverse association was found regardless of hormone receptor-defined subtype or menopausal status.

Conclusions: In this population-based prospective cohort study in Japan we found no association between green tea drinking and risk of breast cancer.

Introduction

Green tea is regularly consumed in Japan and China as a traditional habit and cultural characteristic. Although produced from the same plant, *Camellia sinensis*, differences in the manufacturing process mean that green tea has a higher catechin content than black tea [1-3], which might contribute to its beneficial effects on cancer as well as cardiovascular diseases, and other conditions [3,4]. Specifically, (-)-epigallocatechin-3-gallate (EGCG), the most abundant and biologically active catechin in green tea, might play an important role in cancer prevention [1-3,5,6]. Because breast cancer risk is substantially lower in Asian than Western countries [7], a contribution of high green tea intake to low breast cancer risk has been hypothesized. This hypothesis has been supported by *in vitro* and animal studies, which have demonstrated various protective effects of green tea and tea polyphenols acting via strong antioxidant activity, inhibition of cell proliferation and angiogenesis, induction of apoptosis, and antiestrogenic properties [1-3,5,6,8].

In contrast to *in vitro* and animal studies, few epidemiological studies have examined the association between green tea intake and risk of breast cancer, and their findings have been inconsistent [9-16]. An inverse association was found in three case-control studies among Asian-American and Chinese populations [9-11], whereas no association was observed in two cohort

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studies in Japan [12,13] or one nested case-control study in Singapore [14]. This inconsistency might be in part explained by differences in study design and exposure variation. In the three case-control studies which reported an inverse association, the reference groups were non-green tea drinkers, which included approximately 36 to 68% of control subjects [9-11], whereas in the two Japanese cohort studies the reference groups were women who drank less than one cup per day or one or fewer cups per day [12,13]. Inclusion of green tea drinkers in the reference group might attenuate the difference in risk between the reference and the higher drinking groups. Moreover, the highest consumption group in the two Japanese cohort studies were women who drank five or more cups per day, which included a relatively large portion of subjects (approximately 26 to 43%) [12,13]. Several studies have suggested a possible protective effect of very large amounts of green tea intake: one case-control study in Japan showed a decreased risk of gastric cancer among those who drank 10 or more cups per day [17], for example, while a cohort study in Japan observed a decreased risk of cancer in all sites among those who drank 10 or more cups per day [18]. However, a better understanding of the role of green tea in the etiology of breast cancer would be obtained from studies which included non- to heavy green tea drinkers. In addition, given that *in vitro* studies have suggested the inhibition of aromatase by green tea extracts [8] and of estrogen binding with its receptor by EGCG [6], the effect of green tea intake on the risk of breast cancer may differ according to hormone receptor to defined subtype. Nevertheless, no study has evaluated these associations.

To address these issues, we conducted a large-scale population-based prospective cohort study in Japan on the association between green tea intake and the risk of breast cancer.

Materials and methods

Study population

The Japan Public Health Center-based Prospective Study (JPHC Study), which began in 1990 for Cohort I and in 1993 for Cohort II, included 140,420 inhabitants (68,722 men and 71,698 women) in the municipalities supervised by 11 public health centers (PHC). Details of the study design have been described elsewhere [19]. Study participants were informed about the objectives and methods of the study in written form and those who responded to the survey questionnaire were regarded as consenting to participate in the study. In addition, participants were also notified in writing that they could withdraw from the study. Given no relevant ethical guideline and committee at the time of the survey, the institutional review board of the National Cancer Center, Tokyo, Japan considered return of the questionnaire to be classified as informed consent and approved the study protocol.

The study population comprised registered Japanese inhabitants living in each PHC area, aged 40 to 59 years in Cohort I and 40 to 69 years in Cohort II. In the present analysis, one PHC area was excluded because data on cancer incidence were not available. Thus, after exclusion of ineligible women (n = 98), we defined a population-based cohort of 67,422 women.

Baseline and five-year follow-up survey

Surveys of the cohort were conducted twice by self-administered questionnaire, the baseline in 1990 to 1994 and the five-year follow-up in 1995 to 1998. Of 67,422 women, a total of 55,886 women (83%) returned the baseline questionnaire. Of these, 1,510 who reported a history of cancer in the baseline survey were excluded, leaving 54,376 women as the baseline questionnaire respondents. Of the 67,422 women, we identified 62,788 as eligible for the five-year follow-up survey after exclusion of those who had died, moved out of the study area, or been lost to follow-up before the survey, of whom 52,485 (84%) returned the questionnaire. We also excluded 1,860 women who had a history of cancer at the five-year follow-up survey and 5,813 women who did not respond to the baseline questionnaire, leaving 44,812 women as the five-year follow-up questionnaire respondents.

Exposure measurements

Information on beverages, including green tea, oolong tea, black tea, and coffee, was obtained in the baseline questionnaire in terms of frequency and amount using six precoded categories: less than one cup per week, one to two cups per week, three to four cups per week, and almost daily (further divided into one to two cups per day, three to four cups per day, and five or more cups per day). In the five-year follow-up questionnaire, the consumption of beverages, including two items of green tea, namely *Sencha* (first or second flush of green tea; that is, the first seasonal picking) and *Bancha* (third or fourth flush of green tea; that is, the late seasonal picking)/*Genmaicha* (blend of *bancha* and roasted brown rice), oolong tea, black tea, coffee and canned coffee was assessed in terms of frequency and amount using nine precoded categories: less than one cup per week, one to two cups per week, three to four cups per week, five to six cups per week, one cup per day, two to three cups per day, four to six cups per day, seven to nine cups per day, and ten or more cups per day. *Sencha* and *Bancha* are the two main types of green tea consumed in Japan, and are usually prepared by steeping the tea leaves in hot water. The amounts of *Sencha* or *Bancha*/

Genmaicha consumed (ml per day) were computed by multiplying the frequency by the portion size for each beverage (120 ml per cup). Total green tea intake was defined as the sum of *Sencha* and *Bancha/Genmaicha* intake. The validity of green tea intake reported by the cohort was assessed using dietary records for 28 days (seven-day dietary records in four seasons) or 14 days. Spearman's correlation coefficients for green tea intake between the dietary record data and baseline questionnaire were 0.63 for Cohort I [20] and 0.43 for Cohort II (unpublished data).

Follow-up

All registered women were followed from the start of the study period to 31 December 2006. Data on residential relocation were obtained from residential registries. Among the baseline respondents (n = 54,376), 2,962 women (5.4%) moved out of the study area and 201 (0.4%) were lost to follow-up. The occurrence of cancer was identified by active patient notification from major local hospitals in the study area and data linkage with population-based cancer registries, with permission from the local governments responsible for the registries. Death certificates were used to supplement information on cancer incidence. Site of origin and histologic type were coded by members of our Study Group using the International Classification of Diseases for Oncology, Third Edition (ICD-O-3), code C500-509. Information on estrogen receptor (ER) and progesterone receptor (PR) status was collected from medical records or pathology reports. Up to the end of the study period, 586 new breast cancer cases were identified among the baseline questionnaire respondents (n = 54,376) and 362 cases among the five-year follow-up questionnaire respondents (n = 44,812). Diagnosis was microscopically verified in 95% of 586 cases, and based on death certificates only in 1.0%. Information on ER and PR status was available for 278 (47%) and 262 (45%) of 586 cases, respectively.

Statistical analysis

Since the assessment of green tea intake differed between the baseline and five-year follow-up questionnaires, these were analyzed separately. We excluded 583 women with incomplete information for green tea from 54,376 women, leaving a total of 53,793 women, including 581 breast cancer cases, for inclusion in the baseline data analyses. We also excluded 1,173 women with incomplete information for *Sencha* or *Bancha/Genmaicha* from 44,812 women, leaving a total of 43,639 women, including 350 breast cancer cases, for inclusion in the five-year follow-up data analyses.

In the baseline data analyses, person-years of follow-up were calculated from the baseline survey (1990 to 1994) until the date of diagnosis of breast cancer, date of relocation from the study area, date of death, or end of the study period (31 December 2006), whichever occurred first. Similarly, person-years of follow-up were calculated from the five-year follow-up survey (1995 to 1998) until the date of diagnosis of breast cancer, date of relocation from the study area, date of death, or end of the study period (31 December 2006), whichever occurred first for the five-year follow-up data analyses.

The Cox proportional hazards model was used to estimate hazard ratio (HR) and 95% confidence intervals (CI) of breast cancer by green tea intake using the SAS program (PROC PHREG) (SAS Institute Inc., Cary, NC, USA). The following variables were used for adjustment as potential confounders: age, study area, age at menarche, menopausal status at baseline and age at menopause, number of births, age at first birth, height, body mass index, alcohol intake, smoking status, physical activity, exogenous hormone use, family history of breast cancer, oolong tea intake, black tea intake, coffee intake and canned coffee intake. Linear trends for HRs were tested in the Cox proportional hazards models using the exposure categories as ordinal variables. All P-values reported are two-sided, and significance level was set at $P < 0.05$.

Results

During 733,667 person-years of follow-up (average follow-up, 13.6 years) for 53,793 women between 1990 to 1994 and 2006, a total of 581 cases of breast cancer were newly diagnosed and included in the baseline data analyses; while during 412,801 person-years of follow-up (average follow-up, 9.5 years) for 43,639 women between 1995 to 1998 and 2006, a total of 350 cases of breast cancer were newly diagnosed and included in the five-year follow-up data analyses.

Baseline characteristics of the study participants according to green tea intake are shown in Table 1. Approximately 12% of women drank green tea less than one cup per week while 27% drank five or more cups per day in the baseline data; and 22% and 30% of women did not drink *Sencha* and *Bancha/Genmaicha*, while 5.2% and 2.5% drank 10 or more cups per day, respectively, in the five-year follow-up data. Women who drank five or more cups per day in the baseline data tended to be older, live in towns or villages, be taller, have an earlier onset of first menstruation and fewer births, have a lower use of exogenous female hormones, drink less oolong tea and coffee, and consume more fruits and isoflavones. Similar characteristics was observed for women who drank 10 or more cups of *Sencha* per day in the five-year follow-up data, while women who drank 10 or more cups of *Bancha/Genmaicha* per day tended to be older, have later onset of first

Table 1 Baseline characteristics according to green tea intake*

	Green tea assessed by baseline questionnaire			Green tea assessed by five-year follow-up questionnaire, *Sencha*			Green tea assessed by five-year follow-up questionnaire, *Bancha/Genmaicha*		
	Less than one cup per week	One to two cups per day	Five or more cups per day	Less than one cup per week	Two to three cups per day	Ten or more cups per day	Less than one cup per week	Two to three cups per day	Ten or more cups per day
No. of subjects	6,202	11,322	14,308	9,638	9,052	2,281	13,053	8,148	1,099
Age (year), mean	50.4	50.8	53.9	58.2	57.0	58.9	57.5	57.8	59.7
Residential area (town or village), %	40.9	57.3	68.5	58.7	66.4	73.2	66.9	64.0	60.2
Family history of breast cancer, %	0.7	1.2	1.1	0.9	1.5	0.8	1.3	1.1	0.9
Premenopausal women, %	46	47	31	21	24	15	23	22	14
Age at menopause (year), mean†	49.2	49.3	49.4	-	-	-	-	-	-
Age at menopause (50 to 54 years old), %	-	-	-	46	50	47	47	49	46
Age at menarche (year), mean†	15.0	14.6	14.7	15.0	14.6	14.7	14.7	14.7	14.9
Number of births, mean†	2.9	2.7	2.7	3.0	2.6	2.7	2.7	2.7	2.9
Age at first birth (year), mean†	24.8	25.0	24.9	24.6	25.0	25.0	25.0	24.8	24.6
Use of exogenous female hormones (current use), %	1.4	1.0	1.0	2.9	2.4	2.1	2.4	2.3	2.3
Height (cm), mean†	150.9	152.0	152.2	151.1	152.0	152.3	151.8	151.7	151.9
Body mass index (kg/m2), mean†	23.6	23.3	23.4	23.7	23.3	23.4	23.4	23.4	23.6
Smoking (current smoker), %	7.9	6.5	7.0	5.6	4.4	8.0	6.1	4.1	8.4
Alcohol drinking (regular drinker), %	11	14	11	9.7	13	13	13	11	12
Leisure-time physical activity (≥ once per week), %	15	18	18	-	-	-	-	-	-
Physical activity (metabolic equivalent to hours per day), mean†	-	-	-	32.0	31.9	32.2	31.8	32.1	32.4
Vitamin supplement user, %	20	19	20	12	16	15	15	15	13
Bancha/Genmaicha intake (≥ four cups per day), %	-	-	-	30	6.2	25	-	-	-
Sencha intake (≥ four cups per day), %	-	-	-	-	-	-	43	14	44
Oolong tea intake (≥ one cup per day), %	20	17	10	11	12	8.8	11	13	11
Black tea intake (≥ one cup per day), %	2.8	5.0	3.2	2.6	5.2	3.8	3.3	5.0	4.4
Coffee intake (≥ one cup per day), %	41	49	28	36	40	22	34	40	27
Canned coffee intake (≥ one cup per day), %	-	-	-	4.3	2.7	2.2	2.8	3.2	2.8
Total energy intake (kcal/day), mean†‡	1,770.0	1,764.0	1,805.2	1,770.1	1,923.5	2,194.0	1,833.9	1,934.6	2,185.8
Fish and shellfish intake (g/day), mean†‡	105.5	102.8	105.4	79.6	97.5	114.3	88.2	97.0	105.3
Meats intake (g/day), mean†‡	67.2	65.2	66.1	59.7	60.4	67.7	56.6	61.6	71.6

Table 1 Baseline characteristics according to green tea intake* *(Continued)*

Vegetable intake (g/day), mean†‡	293.8	293.7	295.7	217.0	247.7	319.9	234.9	245.8	327.0
Fruit intake (g/day), mean†‡	175.9	177.3	180.8	208.5	269.9	359.3	243.1	270.1	323.7
Isoflavone intake (mg/day), mean†‡	31.6	32.2	33.5	37.5	43.3	54.7	41.5	42.0	54.8

* Three categories were chosen from six or nine precoded categories, respectively: less than one cup per week, one to two cups per week, three to four cups per week, one to two cups per day, three to four cups per day, and five or more cups per day for baseline questionnaire, and less than one cup per week, one to two cups per week, three to four cups per week, five to six cups per week, one cup per day, two to three cups per day, four to six cups per day, seven to nine cups per day, and ten or more cups per day for five to year follow-up questionnaire.

†Adjusted for age.

‡ Intake for each subject was estimated from the food frequency questionnaires based on a regression function derived from the validation study data (baseline questionnaire only).

menstruation, earlier age at first birth and a larger number of births.

We found no inverse association between green tea intake and the risk of breast cancer regardless of green tea intake as assessed by the baseline or five-year follow-up questionnaire (Table 2). Compared with women who drank less than one cup of green tea per week, the adjusted HR for women who drank five or more cups per day was 1.12 (95% CI 0.81 to 1.56; P for trend = 0.60) in the baseline data analyses. Similarly, compared with women who drank less than one cup of *Sencha* or *Bancha/Genmaicha* per week, adjusted HRs for women who drank 10 or more cups per day in the five-year data analyses were 1.02 (95% CI 0.55 to 1.89; P for trend = 0.48) for *Sencha* and 0.86 (0.34 to 2.17; P for trend = 0.66) for *Bancha/Genmaicha*. Moreover, compared with women who drank neither *Sencha* nor *Bancha/Genmaicha*, the adjusted HR for women who drank more than 1,320 ml per day was 1.29 (0.60 to 2.79; P for trend = 0.70). No substantial change was seen after further adjustment for other potential confounders such as residential area or dietary intake of meat, fish, vegetables, fruit, energy and isoflavones (data not shown). Further, no substantial change was seen in either baseline or five-year follow-up data after the exclusion of women within the first five years of follow-up to minimize the influence of existing preclinical conditions (data not shown), or after the exclusion of women who drank more than one cup of oolong tea or black tea per week to prevent the inclusion of other tea drinkers into the green tea intake reference category (data not shown).

To assess the potential influence of changes in green tea intake during the follow-up period on our findings, we first categorized subjects into three consumption groups for each survey: non-drinkers (corresponding to 'less than one cup per week'), 1 to 719 ml per day, and more than 720 ml per day (corresponding to 'more than five cup per day' in the baseline questionnaire). We next categorized subjects into three groups based on the combination of green tea intake calculated from the both the baseline and five-year follow-up questionnaires: non-drinkers for both questionnaires, more than 720 ml per day for both questionnaires, and other combinations. Compared with women who did not drink green tea for both questionnaires, adjusted HR was 1.12 (95% CI 0.45 to 2.83) for women who drank more than 720 ml per day for both questionnaires.

For analysis of cases by hormone receptor status and among women grouped by menopausal status, we re-categorized women into four groups for the baseline data and three for the five-year follow-up data. In the baseline data analyses, we found no inverse association between green tea intake and the risk of breast cancer regardless of hormone receptor-defined subtype (Table 3). Stratified analyses according to baseline menopausal status showed no remarkable difference between strata. Similar results were obtained when we analyzed the five-year follow-up data (data not shown). Additional stratified analyses according to dietary intake observed no remarkable difference between subgroups defined by dietary isoflavone and folate intake for either the baseline or five-year follow-up data (data not shown).

In additional analyses to investigate the associations of oolong tea, black tea, and coffee intake with breast cancer risk, we found no inverse associations with the baseline (Table 4) or five-year follow-up data (data not shown).

Discussion

In this population-based prospective cohort study, we found no overall association between green tea intake and the risk of breast cancer among Japanese women regardless of menopausal status. Our findings are in general agreement with those of three prospective studies, including two Japanese cohort studies, which found no association between green tea intake and breast cancer risk [12-14]. One noteworthy strength of the present over previous studies is the our remarkably wide variation in green tea intake, from women who drank green

Table 2 Hazard ratio and 95% confidence interval of breast cancer according to green tea intake

	Green tea intake assessed by baseline questionnaire						
	Less than one cup per week	One to two cups per week	Three to four cups per week	One to two cup per day	Three to four cups per day	Five or more cups per day	*P* for trend
No. of cases	68	50	36	117	160	150	
Person-years	87,841	56,200	40,912	152,896	201,005	194,813	
Age- and area-adjusted HR (95% CI)	1.00	1.18 (0.82 to 1.71)	1.17 (0.78 to 1.75)	1.11 (0.82 to 1.50)	1.18 (0.88 to 1.59)	1.12 (0.83 to 1.51)	0.58
Multivariate HR (95% CI)*	1.00	1.19 (0.80 to 1.76)	1.13 (0.72 to 1.75)	1.13 (0.81 to 1.58)	1.17 (0.85 to 1.62)	1.12 (0.81 to 1.56)	0.60
	Green tea intake assessed by five-year follow-up questionnaire						
	Less than one cup per week	One to six cups per week	One cup per day	Two to three cups per day	Four to six cups per day	Seven to nine cups per day	Ten or more cups per day	P for trend
Sencha								
No. of cases	82	56	35	63	78	18	18	
Person-years	91,228	75,237	38,328	85,491	73,160	27,641	21,715	
Age- and area-adjusted HR (95% CI)	1.00	0.79 (0.56 to 1.12)	1.02 (0.68 to 1.52)	0.83 (0.59 to 1.16)	1.22 (0.88 to 1.69)	0.74 (0.44 to 1.26)	0.92 (0.55 to 1.55)	0.77
Multivariate HR (95% CI)†	1.00	0.71 (0.46 to 1.08)	0.97 (0.59 to 1.58)	0.85 (0.57 to 1.27)	1.25 (0.84 to 1.86)	0.68 (0.36 to 1.31)	1.02 (0.55 to 1.89)	0.48
Bancha/Genmaicha								
No. of cases	107	90	38	69	30	7	9	
Person-years	123,189	99,201	42,254	76,532	46,563	14,733	10,329	
Age- and area-adjusted HR (95% CI)	1.00	1.02 (0.77 to 1.36)	1.05 (0.72 to 1.53)	1.09 (0.80 to 1.49)	0.80 (0.53 to 1.21)	0.58 (0.27 to 1.26)	1.02 (0.52 to 2.02)	0.41
Multivariate HR (95% CI)†	1.00	1.12 (0.80 to 1.58)	1.23 (0.79 to 1.92)	1.23 (0.85 to 1.78)	0.83 (0.51 to 1.35)	0.70 (0.28 to 1.76)	0.86 (0.34 to 2.17)	0.66
	Total green tea intake assessed by five-year follow-up questionnaire‡						
	0	1 to 119 ml per day	120 to 299 ml per day	300 to 599 ml per day	600 to 959 ml per day	960 to 1319 ml per day	1320 ml + per day	*P* for trend
No. of cases	13	44	48	85	105	28	27	
Person-years	18,312	50,715	58,499	91,109	114,255	44,745	35,166	
Age- and area-adjusted HR (95% CI)	1.00	1.25 (0.67 to 2.32)	1.25 (0.67 to 2.32)	1.47 (0.81 to 2.67)	1.48 (0.82 to 2.67)	1.02 (0.52 to 2.00)	1.20 (0.61 to 2.36)	0.88
Multivariate HR (95% CI)§	1.00	1.08 (0.52 to 2.23)	1.26 (0.62 to 2.56)	1.59 (0.81 to 3.14)	1.43 (0.73 to 2.83)	0.89 (0.40 to 1.97)	1.29 (0.60 to 2.79)	0.70

,†,§ Adjusted for age (continuous), area (10 public health centers), age at menarche (continuous), menopausal status at baseline (premenopausal women, age at menopause for postmenopausal women (- 47, 48 to 50, 51 to 53, 54+; †,§ - 44, 45 to 49, 50 to 54, 55+)), number of births (0, 1, 2, 3, 4, 5+), age at first birth (-21, 22 to 25, 26 to 29, 30+, nulliparous), height (continuous), BMI (continuous), alcohol intake (non-drinkers, occasional drinkers, -150 (g per week) and 150+ (g per week) among regular drinkers (ethanol)), smoking status (never, past, current), *leisure time physical activity (no, one to three days per month, more than one day per week), †,§ daily physical activity (metabolic equivalent-hours per day) (continuous), exogenous hormone use (never, past, current), family history of breast cancer (yes, no), oolong tea intake (*less than one cup per week, one to four cups per week, one or more cups per day; †,§ less than 1 cup per week, one to six cups per week, one or more cups per day), black tea intake (*less than one cup per week, one to four cups per week, one or more cups per day; †,§ less than one cup per week, one to six cups per week, one or more cups per day), coffee intake (*less than one cup per week, one to four cups per week, one to two cups per day, three or more cups per day; †,§ less than one cup per week, one to six cups per week, one or more cups per day), †,§ canned coffee intake (less than one cup per week, one to six cups per week, one or more cups per day), and †Sencha and Bancha/Genmaicha intake.

Models for Sencha or Bancha/Genmaicha intake did not include these variables, respectively.

‡ Total green tea intake was defined as the sum of Sencha and Bancha/Genmaicha intake (ml per day).

Table 3 Hazard ratio and 95% confidence interval of breast cancer according to subgroup analyses

	Green tea intake assessed by baseline questionnaire								
	Less than one cup per day	One to two cups per day		Three to four cups per day		Five or more cups per day		*P* for trend
All subjects								
No. of cases	154		117		160		150	
Age- and area-adjusted HR (95% CI)	1.00	1.01	(0.79 to 1.29)	1.08	(0.86 to 1.36)	1.02	(0.81 to 1.30)	0.75
Multivariate HR (95% CI)*	1.00	1.04	(0.80 to 1.35)	1.08	(0.84 to 1.39)	1.03	(0.80 to 1.34)	0.76
ER+PR+ breast cancer								
No. of cases	29		25		32		32	
Age- and area-adjusted HR (95% CI)	1.00	1.25	(0.73 to 2.17)	1.18	(0.69 to 2.00)	1.05	(0.62 to 1.79)	0.93
Multivariate HR (95% CI)*	1.00	1.33	(0.75 to 2.38)	1.35	(0.77 to 2.36)	1.02	(0.57 to 1.83)	0.92
ER- PR- breast cancer								
No. of cases	23		16		20		16	
Age- and area-adjusted HR (95% CI)	1.00	1.10	(0.58 to 2.11)	1.09	(0.58 to 2.05)	0.82	(0.42 to 1.60)	0.61
Multivariate HR (95% CI)*	1.00	1.13	(0.55 to 2.32)	1.23	(0.63 to 2.41)	0.81	(0.39 to 1.69)	0.69
Premenopausal women								
No. of cases	81		59		71		51	
Age- and area-adjusted HR (95% CI)	1.00	1.01	(0.72 to 1.43)	1.13	(0.81 to 1.58)	0.95	(0.66 to 1.37)	0.99
Multivariate HR (95% CI)*	1.00	1.05	(0.73 to 1.49)	1.12	(0.79 to 1.58)	0.97	(0.66 to 1.41)	0.99
Postmenopausal women								
No. of cases	70		56		86		96	
Age- and area-adjusted HR (95% CI)	1.00	1.01	(0.71 to 1.45)	1.04	(0.75 to 1.45)	1.05	(0.76 to 1.45)	0.76
Multivariate HR (95% CI)*	1.00	1.01	(0.67 to 1.50)	1.02	(0.71 to 1.48)	1.08	(0.75 to 1.55)	0.67

* Adjusted for age (continuous), area (10 public health centers), age at menarche (continuous), menopausal status at baseline (premenopausal women, age at menopause for postmenopausal women (-47, 48 to 50, 51 to 53, 54+)), number of births (0, 1, 2, 3, 4, 5+), age at first birth (-21, 22 to 25, 26 to 29, 30+, nulliparous), height (continuous), BMI (continuous), alcohol intake (non-drinkers, occasional drinkers, -150 (g/week) and 150+ (g/week) among regular drinkers (ethanol)), smoking status (never, past, current), leisure time physical activity (no, one to three days per month, more than one day per week), exogenous hormone use (never, past, current), family history of breast cancer (yes, no), oolong tea intake (less than one cup per week, one to four cups per week, one or more cups per day), black tea intake (less than one cup per week, one to four cups per week, one or more cups per day) and coffee intake (less than one cup per week, one to four cups per week, one to two cups per day, three or more cups per day).

tea less than one cup per week to those who drank 10 or more cups per day. This strength argues against the possibility that the observed absence of associations with breast cancer risk is attributable to insufficient variation in green tea intake. Our findings therefore suggest that green tea intake within a usual drinking habit is unlikely to reduce the risk of breast cancer.

The other major strength of the present study was its prospective design, in which information was collected before the subsequent diagnosis of breast cancer, thereby avoiding the exposure recall bias inherent to case-control studies. Subjects were selected from the general population, the sample was large, the response rate to the questionnaire (more than 80%) was acceptable for study settings such as this, and the loss to follow-up (0.4%) was negligible. Furthermore, the cancer registry in the study population was of sufficient quality to reduce the possibility of misclassification of the outcome.

Several limitations of this study warrant mention. First, since green tea intake was assessed by self-administered questionnaire, misclassification may have been unavoidable, albeit that our validation study showed relatively high validity [20]. Changes in green tea intake during the follow-up period may also have caused misclassification. To assess the potential influence of this misclassification, we calculated HRs for women who drank more than 720 ml per day in both the baseline and five-year follow-up questionnaires versus those who did not drink green tea in either questionnaire. Although we found no association, we cannot deny the possibility that changes in green tea intake during the follow-up period influenced the findings, particularly considering that the questionnaires used in the baseline and five-year follow-up surveys were different. These misclassifications due to inaccurate measurement would in turn have attenuated the true association, which might be one reason for our results. However, we previously found an inverse association between green tea intake and the risk of distal gastric cancer among women [21], and between green tea intake and the risk of advance prostate cancer among men in the JPHC study using the same analytic approach, namely that exposure status was not updated during follow-up [22]. These findings would also argue against the possibility that the observed absence of associations with breast cancer risk was attributable to inaccurate measurement of green tea intake.

Table 4 Hazard ratio and 95% confidence interval of breast cancer according to oolong tea, black tea and coffee intake as assessed by baseline survey

	Oolong tea intake						
	Less than one cup per week	One to four cups per week		One or more cups per day			*P* for trend
No. of cases	336	153		76			
Person-years	454,964	148,821		93,630			
Age- and area-adjusted HR (95% CI)	1.00	1.37	(1.13 to 1.67)	1.09	(0.85 to 1.41)		0.08
Multivariate HR (95% CI)*	1.00	1.34	(1.09 to 1.66)	0.98	(0.74 to 1.30)		0.40
	Black tea intake						
	Less than one cup per week	**One to four cups per week**		**One or more cups per day**			***P* for trend**
No. of cases	441	111		24			
Person-years	557,038	140,312		24,776			
Age- and area-adjusted HR (95% CI)	1.00	1.00	(0.81 to 1.24)	1.29	(0.85 to 1.96)		0.45
Multivariate HR (95% CI)*	1.00	0.84	(0.67 to 1.07)	1.30	(0.84 to 2.02)		0.80
	Coffee intake						
	Less than one cup per week	**One to four cups per week**		**One to two cups per day**		**Three or more cups per day**	***P* for trend**
No. of cases	161	180		173		63	
Person-years	233,697	213,979		210,969		69,940	
Age- and area-adjusted HR (95% CI)	1.00	1.20	(0.97 to 1.49)	1.18	(0.94 to 1.48)	1.30 (0.95 to 1.77)	0.09
Multivariate HR (95% CI)*	1.00	1.15	(0.91 to 1.46)	1.12	(0.87 to 1.43)	1.22 (0.87 to 1.71)	0.26

* Adjusted for age (continuous), area (10 public health centers), age at menarche (continuous), menopausal status at baseline (premenopausal women, age at menopause for postmenopausal women (-47, 48 to 50, 51 to 53, 54+)), number of births (0, 1, 2, 3, 4, 5+), age at first birth (-21, 22 to 25, 26 to 29, 30+, nulliparous), height (continuous), BMI (continuous), alcohol intake (non-drinkers, occasional drinkers, -150 (g/week) and 150+ (g/week) among regular drinkers (ethanol)), smoking status (never, past, current), leisure time physical activity (no, one to three days per month, more than one day per week), exogenous hormone use (never, past, current), family history of breast cancer (yes, no), green tea intake (less than one cup per day, one to two cups per day, three to four cups per day, five or more cups per day), oolong tea intake (less than one cup per week, one to four cups per week, one or more cups per day), black tea intake (less than one cup per week, one to four cups per week, one or more cups per day) and coffee intake (less than one cup per week, one to four cups per week, one to two cups per day, three or more cups per day). Models for oolong tea, black tea, or coffee intake did not include these variables, respectively.

Second, in spite of a reasonably large cohort population (53,793 women) and long follow-up period (average 13.6 year), the number of breast cancer cases was relatively low (n = 581) in the baseline data analyses, reflecting the low incidence rate in Japan (age-standardized rate per 100,000 world population in 2002, 32.7 in Japan and 101.1 in United States for comparison) [23]. Although we found no association between green tea intake and breast cancer risk (HR for five or more cups per day versus less than one cup per week = 1.12 in the baseline data analyses), the 95% CI was 0.81 to 1.56, which was a relatively wide. Our relatively small sample size therefore cannot deny the possibility of a smaller increase or decrease in risk.

Third, although we measured and adjusted for several potential confounders in the statistical model as far as possible, the effects of confounding by unmeasured variables and residual confounding cannot be totally discarded.

Fourth, information on hormone receptor status was available for 45 to 47% cases in the present study. The major reason for the unknown cases is that collection of this information began in 2002, while data for 1990 to 2002 were obtained by retrospective review of medical records or pathology reports. The relatively small number of cases weakened the statistical power to detect the association. In addition, despite the generally high agreement between the enzyme-linked immunoassay and immunohistochemical techniques, differences in classification as well as interlaboratory variation may result in misclassification, which, if present, may have also attenuated the true association. These methodological limitations might partly explain our findings, which showed no inverse association between green tea intake and the risk of breast cancer regardless of hormone receptor-defined subtype. Thus, further research to clarify the differential associations by hormone receptor-defined subtype is warranted.

Our findings contradict those of three case-control studies, which showed an inverse association [9-11]. Given that all three previous prospective studies showed no association [12-14], these findings might have been influenced by recall and selection bias stemming from the case-control design. Meanwhile, for the three

case-control studies conducted outside Japan [9-11], inconsistent findings might be in part explained by differences in the type of green tea and drinking method. In general, green tea contains catechins, minerals, and vitamins, and their contents vary among green tea types: *Sencha*, for example, one of most popular green teas in Japan, contains higher levels of tannin, vitamin C and folate than *Bancha/Genmaicha* [24]. The present study, however, found no overall association between green tea intake and the risk of breast cancer regardless of type. Moreover, levels of tea polyphenol and other nutrients in green tea varies according to preparation, amount of green tea leaves, frequency of renewing a tea batch in the pot, water temperature, brewing time, and so on. Although two Chinese case-control studies took account of several of these conditions, including green tea leaf amount, brew strength, and tea batch renewal frequency [9,10], no study has directly measured prediagnostic biomarkers of tea polyphenols. In this regard, we conducted a nested cased-control study within the JPHC study and found no overall association between plasma tea polyphenols and the risk of breast cancer [25]. Differences in tea polyphenols level due to green tea type and drinking method are therefore unlikely to explain these inconsistent findings, in any major way at least.

Alternatively, the inconsistencies reported in previous studies might be explained by possible effect modification by dietary factors and genetic polymorphisms [11,14,26]. In a case-control study in Asian-Americans, a risk-reducing effect of green tea was primarily observed among subjects whose soy intake was low [11], while a nested case-control study in Singapore showed a protective effect of green tea against breast cancer among women with high-activity genotypes of the *methylenetetrahydrofolate reductase* (*MTHFR*) and *thymidylate synthase* (*TYMS*) genes [14]. This association was stronger among those whose dietary folate intake was low. Subsequent studies, including our present study, however, have failed to replicate effect modification by soy and folate intake [9,13]. On the other hand, given a lack of information on genetic polymorphisms, the possibility cannot be excluded that green tea drinking is protective among subgroups of women.

Moreover, a recent cohort study in Shanghai showed a time-dependent interaction between green tea consumption and age of breast cancer onset: HRs for women who started green tea drinking at 25 years of age or younger versus non-green tea drinkers were 0.69 for premenopausal breast cancer and 1.61 for postmenopausal breast cancer [16]. This complex observation might also provide an explanation for the inconsistencies reported in previous studies. However, the present study was not able to examine time-dependent interactions because of a lack of information on green tea drinking habits, such as age at the beginning of green tea drinking and years of drinking.

Although oolong tea, black tea, and coffee are less popular than green tea in Japan, they are also rich in phenolic compounds and have been hypothesized to have a protective effect against the development of breast cancer: oolong tea and black tea contain higher levels of theaflavins and thearubigins than catechins, and coffee contains a high level of chlorogenic acid [1-3]. We also found no association of oolong tea, black tea, coffee and canned coffee intake with breast cancer risk. In addition to the relatively small number of women who drank oolong tea, black tea, and coffee, the potential influence of green tea intake on our findings was not fully excluded, albeit that we adjusted for green tea intake. Our findings for black tea, however, are consistent with those of a previous meta-analysis [27]. In contrast, our findings for coffee intake contradict a recent meta-analysis, which suggested a small risk-reducing effect of coffee intake on breast cancer: for example, relative risk (95% CI) per increment of two cups per day was 0.98 (0.96 to 1.00) based on 18 studies [28]. In addition to this small risk reduction, the small variation in coffee intake in our participants might have contributed in part to our findings.

Conclusions

In this population-based prospective cohort study, we found no overall association between green tea intake and the risk of breast cancer among Japanese women. Our findings suggest that drinking green tea as a beverage is unlikely to reduce the risk of breast cancer regardless of green tea type and number of cups within a usual drinking habit.

Abbreviations

CI: confidence interval; EGCG: (-)-epigallocatechin-3-gallate; ER: estrogen receptor; HR: hazard ratio; JPHC Study: Japan Public Health Center-based Prospective Study; PHC: public health centers; PR: progesterone receptor.

Acknowledgements

This study was supported by Grants-in-Aid for Cancer Research and for the Third Term Comprehensive Ten-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan, and Grants-in-Aid for Scientific Research on Priority Areas (17015049) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

We wish to thank all staff members in each study area and in the central offices for their cooperation and technical assistance. We also wish to thank the Iwate, Aomori, Ibaraki, Niigata, Osaka, Kochi, Nagasaki and Okinawa Cancer Registries for their provision of incidence data.

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Competing interests
The authors declare that they have no competing interests.

Received: 21 May 2010 Revised: 12 August 2010
Accepted: 28 October 2010 Published: 28 October 2010

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doi:10.1186/bcr2756
Cite this article as: Iwasaki *et al.*: Green tea drinking and subsequent risk of breast cancer in a population to based cohort of Japanese women. *Breast Cancer Research* 2010 12:R88.

Breast Cancer Res Treat (2010) 124:827–834
DOI 10.1007/s10549-010-0916-x

EPIDEMIOLOGY

Plasma tea polyphenol levels and subsequent risk of breast cancer among Japanese women: a nested case–control study

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Received: 20 April 2010 / Accepted: 22 April 2010 / Published online: 4 May 2010

Abstract Although many in vitro and animal studies have suggested a protective effect of green tea against breast cancer, findings from epidemiological studies have been inconsistent. No study has used prediagnostic biomarkers of tea polyphenols, which might play a protective role. A total of 24,226 women aged 40 to 69 years in the Japan Public Health Center-based Prospective Study who responded to the baseline questionnaire and provided blood in 1990–1995 were followed to December 2002. During a mean 10.6 years of follow-up, 144 newly diagnosed breast cancers were identified. Two matched controls for each case were selected from the cohort. Plasma levels of (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-epigallocatechin-3-gallate (EGCG), and (-)-epicatechin-3-gallate (ECG) were measured, and the odds ratio (OR) of breast cancer according to plasma level was estimated using a conditional logistic regression model. We found no statistically significant association between plasma tea polyphenol levels and breast cancer risk. Adjusted ORs for the highest versus lowest group were 0.90 (95% CI 0.42–1.96; P for trend = 0.98) for EGC, 0.95 (95% CI 0.43–2.08; P for trend = 0.86) for EC, 1.21 (95% CI 0.52–2.80; P for trend = 0.53) for EGCG, and 1.75 (95% CI 0.81–3.78; P for trend = 0.15) for ECG. Stratified analyses according to baseline menopausal status showed no remarkable difference between two strata. This nested case–control study found no overall association between plasma tea polyphenols and the risk of breast cancer in Japan.

Keywords Breast cancer · Plasma tea polyphenol · (-)-Epigallocatechin-3-gallate · Nested case–control study

This study is conducted for the Japan Public Health Center-based Prospective Study Group. The study group members are listed in Appendix.

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Abbreviations

BMI	Body mass index
CI	Confidence interval
EC	(-)-Epicatechin
ECG	(-)-Epicatechin-3-gallate
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin-3-gallate
FFQ	Food frequency questionnaire
JPHC Study	Japan Public Health Center-based Prospective Study
OR	Odds ratio
PHC	Public health centers

Introduction

Green tea is one of the most popular beverages in Japan and China and is regularly consumed as a traditional habit and part of the culture. Due to its high polyphenol content, mainly catechins, there has been considerable interest in the impact of green tea intake on health outcomes, and possible benefits against the risk of cancer, cardiovascular diseases, and other conditions [1, 2]. Today, green tea extracts are a rapidly growing dietary supplement category in the United States.

As breast cancer risk is substantially lower in Asian than Western countries [3], the contribution of a high intake of green tea to low risk of breast cancer has been hypothesized. This hypothesis has been supported by in vitro and animal studies, which together have demonstrated various protective effects of green tea and tea polyphenols acting via strong antioxidant activity, inhibition of cell proliferation and angiogenesis, induction of apoptosis, and antiestrogenic properties [1, 4–7]. To date, however, findings from epidemiological studies have been inconsistent [8–13]. An inverse association was found in three case–control studies based on Asian-American and Chinese populations [8–10], whereas no association was observed in two cohort studies in Japan [11, 12] or one nested case–control study in Singapore [13]. This inconsistency might be in part explained by differences in study design and exposure measurements. Nevertheless, all previous studies assessed green tea intake by self-administered questionnaire or in-person interview [8–13], and no study has directly measured prediagnostic biomarkers of tea polyphenols. Tea polyphenol content in green tea varies according to preparation, type and amount of green tea leaves, frequency of renewing a tea batch in the pot, temperature of the boiled water, and brewing time, among others. To reduce misclassification due to these conditions and to better understand the role of tea polyphenols in the etiology of breast cancer, studies using prediagnostic biomarkers of tea polyphenols are required.

To clarify the effect of tea polyphenols on breast cancer risk, we measured plasma levels of (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-epigallocatechin-3-gallate (EGCG), and (-)-epicatechin-3-gallate (ECG) in a nested case–control study within a large-scale population-based prospective cohort in Japan. These compounds are all characteristic polyphenols, and EGCG in particular is the most abundant and biologically active catechin in green tea. As green tea intake varies widely among individual Japanese, this study provides a special opportunity to better understand the effect of relatively high-dose tea polyphenol levels achievable from habitual tea drinking on breast cancer risk.

Materials and methods

Study population

The Japan Public Health Center-based Prospective Study (JPHC Study), which began in 1990 for Cohort I and in 1993 for Cohort II, included 140,420 subjects (68,722 men and 71,698 women) living in municipalities supervised by 11 public health centers (PHC). Details of the study design have been described elsewhere [14]. The study protocol was approved by the institutional review board of the National Cancer Center, Tokyo, Japan.

The study population comprised registered Japanese residents living in each PHC area, aged 40–59 years in Cohort I and 40–69 years in Cohort II. In the present analysis, one PHC area was excluded since data on cancer incidence were not available. Thus, after exclusion of ineligible subjects ($n = 95$), we defined a population-based cohort of 67,426 women.

Questionnaire survey

A baseline self-administered questionnaire survey was conducted in 1990–1994. A total of 55,891 women (83%) returned the questionnaire, which contained questions concerning demographic characteristics, medical history, menstrual and reproductive history, anthropometric factors, physical activity, smoking and drinking habits, and diet.

Blood collection

Subjects voluntarily provided 10 ml of blood during health checkups in 1990–1995. Blood samples were divided into plasma and buffy layers and stored at −80°C until analysis. Among respondents to the baseline questionnaire, a total of 24,996 women (45%) donated blood.

Follow-up

All registered subjects were followed from the start of the study period to December 31, 2002. Data on residential relocation were obtained from residential registries. Among study subjects ($n = 24,996$), 1,289 subjects (5.2%) moved out of the study area and 5 (0.02%) were lost to follow-up within the study at-risk period.

Selection of cases and controls

Incidence data on breast cancer were collected for the JPHC cancer registry through two data sources, major local hospitals and population-based cancer registries. Death certificates were used to supplement information on cancer incidence. Site of origin and histologic type were coded by

members of our Study Group using the International Classification of Diseases for Oncology, Third Edition, code C500-509. Up to the end of the study period, 144 new breast cancer cases were identified from among the 24,226 women who had returned the baseline questionnaire, did not report a history of breast cancer or ovarian cystoma, and provided blood samples. Diagnosis was microscopically verified in 98% of cases, and based on death certificates only in 0.7%. The mortality/incidence ratio was 0.14.

For each case, two controls were selected using incidence density sampling from subjects who were not diagnosed with breast cancer during the follow-up period when the case was diagnosed. Control selection was done without reference to the incidence of other cancer sites. Controls were matched with each case for age (within 3 years), PHC area, area (city or town and village), date of blood collection (within 90 days), time of day of blood collection (within 3 h), time since last meal at blood collection (within 3 h), and baseline menopausal status.

Laboratory assay

Plasma EGC, EC, EGCG, and ECG were analyzed using high-performance liquid chromatography with a coulometric array detector in accordance with the modified methods of Lee et al. [15, 16]. Concentrations of tea polyphenols were determined by linear regression of the peak height for each standard, and adjusted according to the recovery rate of the internal plasma standard. The regression coefficient of peak height and concentration calculated for tea polyphenols revealed a linearity range 0–0.5 μg/ml, with correlation coefficient values > 0.998. Voltammetric response for the standard solution displayed coefficients of variation of 10% for intra- and 11% for inter-day variation. Recovery rates of tea polyphenols in plasma samples ranged between approximately 63 and 90% (EGC, 90%; EC, 89%; EGCG, 66%; and ECG, 63%). Detection limits were 1.4 ng/ml for EGCG, 1.6 ng/ml for EGC, 1.8 ng/ml for ECG, and 0.3 ng/ml for EC. Laboratory personnel were blinded to case–control status when performing the analyses.

Statistical analysis

Comparison of baseline characteristics between cases and controls was evaluated by the Mantel–Haenszel test using matched-set strata. Spearman's correlation coefficients were calculated between plasma levels and green tea intake assessed using the food frequency questionnaire (FFQ) among control subjects. Using a conditional logistic regression model, we calculated odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer for plasma tea polyphenol levels categorized into three groups based on the control distribution, namely, under the detection limit, and under or over the median level among detected samples. The following variables were adjusted for as potential confounders: age at menarche (continuous), menopausal status at baseline (premenopausal women, age at menopause for postmenopausal women [≤47, 48–50, 51–53, ≥54]), number of births (0, 1, 2, 3, 4, 5+), age at first birth (≤21, 22–25, 26–29, ≥30, nulliparous), height (continuous), body mass index (BMI) (continuous), alcohol intake (non-drinkers, occasional drinkers, 50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol)). Linear trends for ORs were tested in the conditional logistic regression model using the exposure categories as ordinal variables. To perform stratified analyses according to menopausal status, levels of tea polyphenols in the plasma were dichotomized into the "detected" and "not detected" categories. Since previous studies have suggested effect modification of green tea intake by dietary factors [10, 13], we further performed stratified analyses by plasma genistein level, and dietary isoflavone and folate intake. All p values reported are two-sided, and significance level was set at $P < 0.05$. All statistical analyses were performed with SAS software version 9.1 (SAS Institute, Inc., Cary, NC).

Results

Case subjects and controls had a significantly different distribution for number of births (Table 1). Other characteristics, such as age at menarche, age at first birth, BMI, alcohol drinking, and dietary intake, did not substantially differ between the two groups.

The proportion of subjects in whom tea polyphenols were not detected was 69% for EGC, 76% for EC, 80% for EGCG, and 81% for ECG. Correlation coefficients between plasma levels and green tea intake were low, at 0.23 for EGC, 0.16 for EC, 0.18 for EGCG, and 0.17 for ECG. We found no statistically significant association between plasma tea polyphenol level and the risk of breast cancer (Table 2). Adjusted ORs for the highest versus lowest group were 0.90 (95% CI 0.42–1.96; P for trend = 0.98) for EGC, 0.95 (95% CI 0.43–2.08; P for trend = 0.86) for EC, 1.21 (95% CI 0.52-2.80; P for trend = 0.53) for EGCG, and 1.75 (95% CI 0.81–3.78; P for trend = 0.15) for ECG. In addition to each tea polyphenol, total plasma tea polyphenol level, defined as the sum of plasma EGC, EC, EGCG, and ECG levels, was not associated with the risk of breast cancer (data not shown). No substantial change was seen after further adjustment for other potential confounders, namely family history of breast cancer, use of exogenous female hormones, leisure-time physical activity, smoking habit, and dietary intake of meat, fish, vegetables, fruit, energy and isoflavones (data not shown). Further,

Table 1 Characteristics of case and matched control subjects at baseline

	Case (n = 144)	Control (n = 288)	P[a]
Age (year), mean (SE)	52 (0.6)	52 (0.4)	-
Family history of breast cancer, n (%)	2 (1)	2 (0.7)	0.48
Premenopausal women, n (%)	59 (42)	118 (42)	-
Age at menopause (year), mean (SE)[b]	50 (0.4)	50 (0.3)	0.76
Age at menarche (year), mean (SE)[b]	15 (0.1)	15 (0.1)	0.33
Number of births, mean (SE)[b]	2 (0.1)	3 (0.1)	0.01
Age at first birth (year), mean (SE)[b]	26 (0.3)	25 (0.2)	0.22
Use of exogenous female hormones (current use), n (%)	4 (3)	2 (0.8)	0.10
Height (cm), mean (SE)[b]	152 (0.5)	151 (0.3)	0.70
Body mass index (kg/m^2), mean (SE)[b]	23 (0.3)	24 (0.2)	0.49
Smoking (current smoker), n (%)	5 (3)	17 (6)	0.23
Alcohol drinking (regular drinker), n (%)	18 (13)	26 (9)	0.28
Leisure-time physical activity (≥once per week), n (%)	30 (21)	57 (20)	0.42
Vitamin supplement user, n (%)	33 (24)	61 (23)	0.65
Green tea intake (≥five cups per day), n (%)	36 (25)	71 (25)	0.42
Coffee intake (≥five cups per day), n (%)	4 (3)	5 (2)	0.83
Total energy intake (kcal/day), mean (SE)[b,c]	1,810 (12)	1,807 (8)	0.41
Fish and shellfish intake (g/day), mean (SE)[b,c]	109 (1)	110 (0.8)	0.75
Meat intake (g/day), mean (SE)[b,c]	68 (0.5)	68 (0.4)	0.15
Vegetable intake (g/day), mean (SE)[b,c]	297 (1)	296 (0.8)	0.20
Fruit intake (g/day), mean (SE)[b,c]	181 (2)	180 (1)	0.79
Isoflavone intake (mg/day), mean (SE)[b,c]	33 (0.7)	33 (0.5)	0.30

[a] P for Mantel–Haenszel test with matched-set strata

[b] Adjusted for age

[c] Intake for each subject was estimated from the food frequency questionnaires based on a regression function derived from the validation study data

exclusion of subjects who provided a non-fasting blood sample, i.e., within 6 h after a meal, or who reported a history of chronic disease did not substantially change the results (data not shown). On the other hand, non-significant inverse associations were observed for plasma EGC and EC level when cases diagnosed before the first 4 years of follow-up were excluded, with adjusted ORs for the highest versus lowest group of 0.42 (95% CI 0.15–1.16; P for trend = 0.07) for EGC and 0.36 (95% CI 0.12–1.12; P for trend = 0.06) for EC.

Stratified analyses according to baseline menopausal status showed no remarkable difference between two strata regardless of tea polyphenol level (Table 3). In addition, no differential association was observed between subgroups defined by plasma genistein level, and dietary isoflavone and folate intake (data not shown).

Discussion

In this nested case–control study, we found no overall association between plasma tea polyphenols and the risk of breast cancer among Japanese women. Our findings are in general agreement with those of three prospective studies, which found no association between green tea intake and the risk of breast cancer [11–13], but contradict those of three case–control studies, which showed an inverse association [8–10]. Owing to a difference in exposure assessments between plasma tea polyphenols and green tea drinking habit, direct comparison of these results is difficult. Plasma tea polyphenol levels are partly determined by individual differences in absorption and metabolism, in addition to several conditions related to methods of drinking green tea, as mentioned in the Introduction. In fact, correlation coefficients between plasma levels and green tea intake were low, ranging between 0.16 for EC and 0.23 for EGC. For these reasons, plasma levels might better reflect interpersonal differences than green tea drinking habit. In this regard, our findings, based on such direct measurement, suggest that tea polyphenols at the levels measured in plasma might not play a major role in the etiology of breast cancer.

The observed absence of associations with breast cancer risk might be attributable to either or both poor

Table 2 Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to plasma tea polyphenol levels

	Categories			*P* for trend
	Not detected	Under median value[a]	Over median value[a]	
EGC (ng/ml)				
Median[b]	Not detected	27.6	189.7	
No. of cases/no. of controls	97/202	28/43	19/43	
OR (95% CI)	1.00	1.40 (0.79–2.47)	0.90 (0.45–1.78)	0.87
Multivariate OR (95% CI)[c]	1.00	1.29 (0.65–2.54)	0.90 (0.42–1.96)	0.98
EC (ng/ml)				
Median[b]	Not detected	13.1	66.3	
No. of cases/no. of controls	109/221	19/33	16/34	
OR (95% CI)	1.00	1.20 (0.61–2.36)	0.97 (0.49–1.91)	0.96
Multivariate OR (95% CI)[c]	1.00	0.93 (0.43–2.02)	0.95 (0.43–2.08)	0.86
EGCG (ng/ml)				
Median[b]	Not detected	16.9	58.9	
No. of cases/no. of controls	113/233	16/27	15/28	
OR (95% CI)	1.00	1.25 (0.62–2.51)	1.12 (0.55–2.25)	0.61
Multivariate OR (95% CI)[c]	1.00	1.27 (0.58–2.80)	1.21 (0.52–2.80)	0.53
ECG (ng/ml)				
Median[b]	Not detected	7.1	14.7	
No. of cases/no. of controls	113/237	12/25	19/26	
OR (95% CI)	1.00	1.06 (0.49–2.29)	1.59 (0.82–3.08)	0.20
Multivariate OR (95% CI)[c]	1.00	1.28 (0.51–3.21)	1.75 (0.81–3.78)	0.15

EGC (-)-epigallocatechin, *EC* (-)-epicatechin, *EGCG* (-)-epigallocatechin-3-gallate, *ECG* (-)-epicatechin-3-gallate

[a] Among detected samples

[b] Median plasma level among control group for EGC, EC, EGCG, ECG

[c] Adjusted for age at menarche (continuous), menopausal status at baseline (premenopausal women, age at menopause for postmenopausal women [–47, 48–50, 51–53, 54+]), number of births (0, 1, 2, 3, 4, 5+), age at first birth (–21, 22–25, 26–29, 30+, nulliparous), height (continuous), BMI (continuous), alcohol intake (non-drinkers, occasional drinkers, 50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol)). Adjusted ORs were calculated based on a total of 387 subjects with complete information of covariates

bioavailability or errors in the measurement of tea polyphenols. In fact, a large proportion of subjects had no detectable plasma level of tea polyphenols [17] despite a high and large variation in green tea intake: for example, only 15% of control women did not drink green tea, whereas 69% drank daily, of whom 36% drank more than five cups per day. However, we previously found an inverse association between plasma tea polyphenols, namely plasma ECG level, and the risk of gastric cancer among women in a nested case–control study within the JPHC study [18], which used the same population and method for measuring plasma tea polyphenols as the present study. This finding would argue against the possible explanation for the lack of association as being due to poor bioavailability and measurement error. Meanwhile, given that at least 20% of our subjects had detectable plasma levels of tea polyphenols despite their poor bioavailability, Japanese might represent a unique population in which to evaluate the effect of tea polyphenols on the risk of breast cancer. Our findings therefore suggest that tea polyphenols are unlikely to reduce the risk of breast cancer even at relatively high detectable concentrations achievable from habitual green tea drinking only.

One possible concern is that the long storage of blood samples might have lead to a large proportion of subjects having no detectable plasma levels of tea polyphenols due to degradation in plasma. No data for the effect of storage time on plasma level are available. In the present study, blood samples of cases and controls were stored under the same conditions. In addition, we matched for date of blood collection between cases and controls and measured plasma levels in the same batch to minimize measurement errors. Degradation of tea polyphenols in plasma is therefore unlikely to explain the observed absence of associations with breast cancer risk.

We found non-significant inverse associations between plasma EGC and EC level and the risk of breast cancer when cases diagnosed before the first 4 years of follow-up were excluded. The exclusion of these subjects might have minimized the possible influence of existing diseases and

Table 3 Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to plasma tea polyphenol levels by baseline menopausal status

	Categories	
	Not detected	Detected
Premenopausal women		
EGC		
No. of cases/no. of controls	40/85	19/33
Multivariate OR (95% CI)[a]	1.00	1.44 (0.58–3.58)
EC		
No. of cases/no. of controls	46/91	13/27
Multivariate OR (95% CI)[a]	1.00	1.15 (0.43–3.11)
EGCG		
No. of cases/no. of controls	46/99	13/19
Multivariate OR (95% CI)[a]	1.00	1.78 (0.66–4.79)
ECG		
No. of cases/no. of controls	47/100	12/18
Multivariate OR (95% CI)[a]	1.00	1.67 (0.62–4.50)
Postmenopausal women		
EGC		
No. of cases/no. of controls	52/108	28/52
Multivariate OR (95% CI)[b]	1.00	0.95 (0.42–2.18)
EC		
No. of cases/no. of controls	58/120	22/40
Multivariate OR (95% CI)[b]	1.00	1.11 (0.43–2.84)
EGCG		
No. of cases/no. of controls	62/124	18/36
Multivariate OR (95% CI)[b]	1.00	1.22 (0.50–2.95)
ECG		
No. of cases/no. of controls	61/127	19/33
Multivariate OR (95% CI)[b]	1.00	1.91 (0.72–5.07)

EGC (-)-epigallocatechin, *EC* (-)-epicatechin, *EGCG* (-)-epigallocatechin-3-gallate, *ECG* (-)-epicatechin-3-gallate

[a] Adjusted for age at menarche (continuous), number of births (0, 1, 2, 3, 4, 5+), age at first birth (–21, 22–25, 26–29, 30+, nulliparous), height (continuous), BMI (continuous), alcohol intake (non-drinkers, occasional drinkers, 50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol))

[b] Adjusted for age at menarche (continuous), age at menopause (–47, 48–50, 51–53, 54+), number of births (0, 1, 2, 3, 4, 5+), age at first birth (–21, 22–25, 26–29, 30+, nulliparous), height (continuous), BMI (continuous), alcohol intake (non-drinkers, occasional drinkers, 50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol))

their symptoms on green tea drinking habit, other lifestyle factors, and health behavior. Meanwhile, no association was observed when subjects who reported a history of chronic disease were excluded, indicating that the present findings were unlikely to have been affected by a history of chronic diseases. Moreover, it is unlikely that subjects changed their dietary habits, particularly green tea drinking habit, due to the presence of subclinical breast cancer. Given the relatively small number of cases in this study, these findings might rather be due to chance.

The association between green tea drinking and the risk of breast cancer may differ by menopausal status. A large population-based case–control study in Shanghai (n = 3454 cases), however, showed a lower risk of breast cancer among regular green tea drinkers than non-drinkers for both pre- and postmenopausal women [8]. Further, our study observed no association regardless of menopausal status. On the other hand, several previous studies have suggested effect modification of green tea intake by dietary factors and genetic polymorphisms [10, 13]. In a case–control study in Asian-Americans, a risk-reducing effect of green tea was primarily observed among subjects whose soy intake was low [10], while a nested case–control study in Singapore showed a protective effect of green tea against breast cancer among women with high-activity genotypes of the *methylenetetrahydrofolate reductase* (*MTHFR*) and *thymidylate synthase* (*TYMS*) genes [13]. This association was stronger among those whose dietary folate intake was low. Subsequent studies, however, have failed to replicate effect modification by soy and folate intake [8, 12]. Similarly, our stratified analyses suggested no differential association between subgroups defined by plasma genistein level, or dietary isoflavone and folate intake.

Our study has several methodological advantages over previous studies of green tea drinking and the risk of breast cancer. First, this is the first epidemiological study to directly measure tea polyphenols in plasma samples as an exposure assessment, these being the substances in green tea which putatively exert protective effects against breast cancer. Their direct measurement may allow us to elucidate the mechanisms by which green tea might influence breast cancer development. Second, since blood samples were collected before cancer diagnosis in our nested case–control study within a prospective cohort, any potential bias due to the presence of cancer is likely obviated. Third, cases and controls were selected from the same cohort, thereby avoiding the selection bias inherent to case–control studies.

Several limitations of this study also warrant mention. First, we measured tea polyphenols in plasma samples only once for each individual. Green tea drinking is a personal habit, and consumption levels of most individuals are assumed to be relatively stable over time in Japan, as suggested by our validation study [19, 20], which showed relatively high reproducibility of repeated measurements of green tea intake by FFQ (correlation coefficient = 0.64 for one-year interval and 0.54 for five-year interval) (unpublished data). By comparison, pharmacokinetic studies in humans have shown that the half-life of EGCG in blood ranges from 2 to 5 h [17], and that plasma levels are particularly affected by time elapsed since the last meal. To minimize the attenuation of risk estimates derived from random

measurement errors, we therefore matched fasting time between cases and controls. Second, in spite of a reasonably large cohort population (24,226 women) and long follow-up period (average 10.6 year), the number of breast cancer cases was relatively small, reflecting the low incidence rate in Japan (age-standardized rate per 100,000 world population in 2002, 32.7 in Japan and 101.1 in United States for comparison) [21]. The interpretability of our results might therefore be limited, particularly in stratified analyses. Third, although our cohort subjects were selected from the general population, subjects were restricted to the 24,226 women (43%) respondents to the baseline questionnaire who provided blood samples. Thus, any extrapolation of the results to the general population should be done cautiously, particularly in view of a previous report showing the difficulty of extrapolating relative risk estimates for a sub-cohort to an entire cohort. This difficulty might in fact be inherent to prospective studies in general [22].

Allowing for these methodological issues, we found no overall association between plasma tea polyphenols and the risk of breast cancer in a nested case–control study in Japan. This finding suggests that tea polyphenols are unlikely to reduce the risk of breast cancer substantially, even at relatively high detectable concentrations.

Acknowledgments This study was supported by Grants-in-Aid for Cancer Research and for the Third Term Comprehensive Ten-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan, and Grants-in-Aid for Scientific Research on Priority Areas (17015049) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We wish to thank all staff members in each study area and in the central offices for their cooperation and technical assistance. We also wish to thank the Iwate, Aomori, Ibaraki, Niigata, Osaka, Kochi, Nagasaki, and Okinawa Cancer Registries for their provision of incidence data.

Conflict of interest statement All of the authors declare that they have no conflict of interest in connection with this paper.

Appendix

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