Research Article

Plasma Isoflavones and Risk of Primary Liver Cancer in Japanese Women and Men with Hepatitis Virus Infection: A Nested Case–Control Study

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Abstract

Background: Evidence suggests that estrogen plays a preventive role in primary liver cancer development, and it might be thought that isoflavones, which are structurally similar to estrogens and bind to estrogen receptors, are associated with the risk of liver cancer. We investigated this suspected association by measuring plasma concentrations of isoflavones in a nested case–control study of a population-based prospective cohort in Japan.

Methods: From 18,628 target participants ages 40 to 69 years who returned the baseline questionnaire and provided blood samples, we selected those with either hepatitis B or hepatitis C virus infection at baseline ($n = 1,544$). Among these, 90 (28 women and 62 men) were newly diagnosed with primary liver cancer from 1993 through 2006; they were matched with 175 controls (54 women and 121 men). Plasma concentrations of isoflavones (genistein, daidzein, glycitein, and equol) were mea-

sured using triple quadrupole tandem liquid chromatography-mass spectrometry. The ORs of liver cancer development based on plasma concentrations were estimated with a conditional logistic regression model.

Results: Basically, distributions of plasma isoflavone concentrations did not differ between the cases and controls. No statistically significant associations of genistein, daidzein, glycitein, and equol with primary liver cancer risk were found in either women or men.

Conclusions: In middle-aged Japanese women and men with hepatitis virus infection, plasma isoflavones were unassociated with the occurrence of primary liver cancer.

Impact: The role of isoflavones in liver carcinogenesis merits further study using both biomarkers and data on dietary intake of isoflavones. *Cancer Epidemiol Biomarkers Prev; 24(3); 532–7. ©2014 AACR.*

Introduction

Women worldwide have a lower incidence of primary liver cancer, respond better to treatment, and show better survival (1). These data indicate that estrogen plays a preventive role in liver cancer development. In sex hormone–related cancers such as breast cancer, an association is suspected between isoflavones and cancer risk, because isoflavones are structurally similar to 17β-estradiol, have the ability to bind to estrogen receptors, and act not only as estrogen agonists but also as antagonists (2). We previously examined the association between dietary intake of isoflavones and primary liver cancer, and found that isoflavone consumption was positively associated with liver cancer risk among Japanese women (3). Given the preventive effects of estrogen against liver cancer, we thought this positive association might be partly explained by the antiestrogenic effects of isoflavones. The main exposure variable in our previous study was isoflavone consumption as assessed via a food-frequency questionnaire, so clearly a more objective measure was required to confirm the association epidemiologically. Isoflavone concentrations in the blood are superior to dietary assessments as markers reflecting *in vivo* absorption and metabolism (4).

In this study, we examined the effects of plasma isoflavone concentrations on primary liver cancer risk among women and men with hepatitis virus infection, using a nested case–control design based on data from a large-scale population-based prospective cohort study in Japan. As far as we know, no previous prospective studies have examined liver cancer using biomarkers to assess isoflavone exposure.

Materials and Methods

Study population

The study design of the Japan Public Health Center-based Prospective Study (JPHC Study), which began in 1990 for

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

Note: Study group members are listed in the Acknowledgments section.

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cohort I and in 1993 for cohort II, has been published elsewhere (5). Cohort I included all registered Japanese residents ages 40 to 59 years of 5 public health center areas, and in cohort II, all residents ages 40 to 69 years of 6 other areas. The present study was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan.

In this study, we used cohort II data. In cohort II (1993–1994), 56,542 participants (response rate, 82%) answered a baseline questionnaire on sociodemographic characteristics, medical history, smoking and drinking habits, diet, and so on. Of these, 37% voluntarily provided 10 mL of blood at health checkups during the baseline survey (1993–1995). The blood samples were divided into plasma and buffy layers and preserved at −80°C until analysis. We measured hepatitis B surface antigen (HBsAg) by reversed passive hemagglutination with a commercial kit (Institute of Immunology Co., Ltd.) and anti-hepatitis C virus antibody (anti-HCV) with a third-generation immunoassay (Lumipulse II Ortho HCV; Ortho-Clinical Diagnosis K.K.). Study participants were informed of the objectives and methods of the study in writing, and those who answered the questionnaire and donated blood were regarded as having given informed consent to participate. Of these, we selected only those who had no history of cancer at baseline and had provided data on basic characteristics, leaving us with a total of 18,628 participants (6,401 men and 12,227 women).

Follow-up

Participants were followed up from the date of blood collection until December 31, 2006. Information on residence status and survival was obtained annually through residential registries. With a follow-up rate of 99.7%, selection bias due to lost to follow-up was negligible.

Data on primary liver cancer incidence were collected for the JPHC cancer registry from two data sources: major local hospital records and population-based cancer registries. Cases were coded using the International Classification of Diseases for Oncology, Third Edition (code: C22.0; ref. 6). Death certificates were used as a supplementary information source. The proportion of cases for which information was available from death certificates only was 4.7%, indicating satisfactory cancer registry system quality during the study period.

Selection of cases and controls

From the 18,628 participants, we selected 1,544 infected either with hepatitis B virus (HBV; positivity for HBsAg) or hepatitis C virus (HCV; positivity for anti-HCV) at baseline. Up to the end of the study period after blood collection, we identified 91 new cases of primary liver cancer among these 1,544 participants. For each case, we selected 2 controls at random from among the participants with no history of liver cancer when the case was diagnosed. Controls were matched to each patient with respect to age (within 5 years), sex, public health center area, fasting status at blood collection, hepatitis virus infection status (HBV or HCV), and baseline menopausal status (for women). We could not find appropriate matched controls for 1 patient, and found only 1 matched control with a sufficient quantity of plasma for each of 5 other patients. Finally, a total of 90 patients (28 women and 62 men) and 175 controls (54 women and 121 men) were included in the present analysis.

Laboratory assay for isoflavones

From the blood samples collected at baseline, plasma concentrations of isoflavones (genistein, daidzein, glycitein, and equol) were assessed using triple quadrupole tandem liquid chromatography-mass spectrometry (7). All samples were analyzed at a single laboratory (SRL). Laboratory technicians performed the analyses under the mask of case–control status, and samples from matched sets were assayed together. The detection limit for all of the isoflavones was 1.0 ng/mL. For quality control, a pooled blood sample from healthy volunteers was used, and interassay and intraassay coefficients of variation were <6.2% and <3.0% for all isoflavones, respectively.

Statistical analysis

Using our previous findings (3), we performed sex-specific analyses. Comparisons of the baseline characteristics between the cases and controls were performed with the χ^2 or the Mann–Whitney test, as appropriate. In the controls, Spearman rank correlation coefficients were calculated for plasma concentrations and dietary intakes of genistein and daidzein. The dietary genistein and daidzein intake as assessed with the food-frequency questionnaire has been described in detail previously (3).

Using a conditional logistic regression model, we calculated ORs and 95% confidence intervals (CI) of primary liver cancer development for plasma genistein and daidzein concentrations divided into sex-specific tertiles according to the frequency of distribution among the controls. For glycitein and equol concentrations, three categories were defined: participants with concentrations below the detection limit, and lower half and upper half of those above the detection limit. The trend was tested by assigning ordinal values for categorical variables. In a multivariable model, we adjusted for the following variables previously associated with liver cancer risk (8): alcohol consumption (never, past, or regular for women, and never, past, <150, 150 to <450, or ≥450 g/week ethanol for men); body mass index (BMI; <25.0, ≥25.0 kg/m^2); diabetes defined as a self-reported history of diabetes, and/or antidiabetic medication use, and/or blood glucose ≥5.55 mmol/L (100 mg/dL) fasting or ≥7.77 mmol/L (140 mg/dL) nonfasting (yes, no); and coffee consumption (almost never, once a week to <1 cup/day, ≥1 cup/day). An additional model was further adjusted for serum alanine aminotransferase (ALT) levels (<30, 30–69, ≥70 IU/L; ref. 9). We also entered the following variables in the model: smoking status (a suspected risk factor for liver cancer), vegetable intake, fish intake, and plasma concentrations of total adiponectin (factors associated with liver cancer risk in the JPHC Study; refs. 10–12). However, the inclusion of these factors did not change the risk estimates substantially.

Subgroup analyses were performed for 79 patients with cancer with HCV infection (26 women and 53 men) and 27 female patients after menopause at baseline. To examine the effect modification of exposure to isoflavones by BMI (<25.0, ≥25.0 kg/m^2) and diabetes (yes or no), factors associated with both isoflavones (13) and liver cancer (8), we conducted stratified analyses using an unconditional logistic regression model adjusted for matching factors and variables in the multivariable model. In addition, stratified analyses of equol producers (defined as participants with equol concentrations above the detection limit of ≥ 1.0 ng/mL) were performed, because the beneficial health effects of isoflavones were likely to differ between equol producers with specific intestinal bacteria and nonproducers (14). In these stratified analyses, we dichotomized participants into low and

Table 1. Selected baseline characteristics of cases and controls

	Women			Men		
	Cases (*n* = 28)	Controls (*n* = 54)		Cases (*n* = 62)	Controls (*n* = 121)	
Variables	Prevalence (%)	Prevalence (%)	*P* value[a]	Prevalence (%)	Prevalence (%)	*P* value[a]
Age, y						
40–49	3.6	3.7	Matching variable	1.6	3.3	Matching variable
50–59	25.0	27.8		29.0	28.9	
60–69	71.4	68.5		69.4	67.8	
Hepatitis virus infectious status[b]						
HBV	7.1	5.6	Matching variable	14.5	14.0	Matching variable
HCV	92.9	94.4		85.5	86.0	
Menopausal status, premenopausal	3.6	3.7	Matching variable			
Alcohol consumption, regular drinker	10.7	25.9	0.14	50.0	66.9	0.08
Smoking status, current smoker	14.3	7.4	0.08	48.4	47.9	0.81
BMI, ≥25 kg/m^2	46.4	22.2	0.02	33.9	14.9	<0.01
Diabetes, yes[c]	17.9	11.1	0.40	40.3	27.3	0.07
Coffee consumption, ≥1 cup/day	25.0	35.2	0.35	22.6	40.5	0.02
ALT level, ≥70 IU/L	53.6	4.0	< 0.01	42.4	7.0	<0.01
Vegetable intake (g/day)[d]	43.3 (32.5–66.0)	49.1 (30.9–71.5)	0.65	48.1 (25.3–75.8)	48.7 (30.5–75.3)	0.48
Fish intake (g/day)[d]	37.7 (22.8–57.4)	38.1 (21.0–53.5)	0.93	58.9 (37.5–76.0)	52.5 (32.7–73.2)	0.40
Dietary intake of genistein (mg/day)[d]	14.0 (10.5–20.5)	10.3 (6.4–18.7)	0.01	11.9 (6.6–21.2)	13.6 (8.2–20.4)	0.63
Dietary intake of daidzein (mg/day)[d]	8.4 (6.3–12.3)	6.1 (3.8–10.0)	0.01	7.1 (3.9–12.9)	8.1 (4.9–12.2)	0.63

[a]Calculated using the χ^2 test and the Mann–Whitney test.
[b]Positive for hepatitis B surface antigen was regarded as indicating HBV infection and positive for anti-hepatitis C virus antibody as indicating HCV infection.
[c]Diabetes was defined as a self-reported history of diabetes, and/or antidiabetic medication use, and/or blood glucose ≥5.55 mmol/L (100 mg/dL) fasting or ≥7.77 mmol/L (140 mg/dL) nonfasting.
[d]Energy-adjusted by using the residual method, median (interquartile range).

high groups, based on the median concentrations of genistein and daidzein in the controls. For glycitein and equol, participants were categorized into low (not detected) and high (detected) groups. We used a likelihood ratio test to examine the potential effect modifications according to the stratified variables.

All analyses were performed with STATA version 11 (STATA Corporation, College Station). All *P* values reported are two sided, and differences at *P* < 0.05 were considered significant.

Results

The baseline characteristics of the case and control groups are shown in Table 1. Among the women, the proportions of overweight and high ALT levels and dietary intakes of genistein and daidzein were higher in the case group than in the control group. Among the men, there were statistically different distributions of BMI, coffee consumption, and ALT levels between the case and control groups. The groups showed no differences in median plasma concentrations of isoflavones, except for equol in women: median concentrations of equol tended to be lower in the cases than in the controls (0 vs. 2.8 ng/mL, *P* = 0.04; Table 2). Spearman rank correlation coefficients between plasma concentrations and dietary intake of genistein were 0.12 for women and 0.27 for men, and those of diadzein were 0.09 for women and 0.29 for men.

We found no consistent association in either sex between plasma isoflavone concentrations and primary liver cancer risk

(Table 3). The multivariable ORs of primary liver cancer for the high versus low tertiles of genistein, daidzein, glycitein, and equol were 1.31 (95% CI, 0.28–6.05), 0.55 (0.10–3.19), 1.97 (0.35–10.93), and 0.44 (0.13–1.49), respectively, for women; for men, they were 1.33 (0.58–3.09), 1.23 (0.47–3.21), 2.14 (0.82–5.59), and 1.35 (0.60–3.04). Further adjustment for ALT levels did not change the tendency of the results (data not shown). No material change was seen when the premenopausal women were excluded: multivariable ORs for the high versus low group are 1.12 (95% CI, 0.24–5.35) for genistein, 0.50 (0.09–2.89) for diadzein, 1.79 (0.32–10.14) for glycitein, and 0.37 (0.10–1.34) for equol. We also observed a null association when we restricted analyses to participants with HCV infection (data not shown). In addition, there was no statistical evidence of any effect modification across the strata of BMI, diabetes, and equol producers (Supplementary Table S1).

Discussion

Our previous cohort analysis showed that dietary intake of isoflavones increased the risk of primary liver cancer in women (3). Multivariable HRs for the high versus low tertiles of genistein intake and daidzein intake were 3.19 (95% CI, 1.13–9.00, *P* for trend = 0.03) and 3.90 (1.30–11.69, *P* for trend = 0.01), respectively. Even when analysis was restricted to participants infected with hepatitis virus (25 cases of liver cancer), this positive

Table 2. Plasma concentrations of isoflavones in cases and controls

	Women			Men		
	Cases (*n* = 28)	Controls (*n* = 54)		Cases (*n* = 62)	Controls (*n* = 121)	
	Median (interquartile	Median (interquartile		Median (interquartile	Median (interquartile	
Isoflavones	range)	range)	*P* value[a]	range)	range)	*P* value[a]
Genistein (ng/mL)	46.5 (16.2–111.4)	44.6 (17.1–164.2)	0.71	94.8 (30.2–201.9)	64.7 (31.3–162.5)	0.25
Diadzein (ng/mL)	18.8 (3.3–43.9)	21.7 (5.3–65.0)	0.30	31.8 (7.3–81.2)	27.4 (10.0–66.8)	0.76
Glycitein[b] (ng/mL)	1.5 (0–3.5)	1.1 (0–3.5)	0.77	2.4 (0–7.8)	2.1 (0–5.4)	0.22
Equol[b] (ng/mL)	0 (0–3.3)	2.8 (0–14.6)	0.04	7.1 (0–26.9)	3.7 (0–16.7)	0.25

[a]Calculated using the Mann–Whitney test.
[b]Values below the detection limit (< 1.0 ng/mL) were regarded as zero.

Table 3. ORs and 95% CIs of primary liver cancer according to plasma concentrations of isoflavones

Plasma concentration	Women				Men			
	Low	Middle	High	P for trend[a]	Low	Middle	High	P for trend[a]
Genistein (ng/mL)	<28.0	28.0–100.9	>100.9		<41.9	41.9–111.0	>111.0	
Number of cases/controls	11/18	9/18	8/18		21/41	14/40	27/40	
Matching variables adjusted OR (95% CI)[b]	1.00 (reference)	0.79 (0.27–2.33)	0.65 (0.18–2.29)	0.50	1.00 (reference)	0.70 (0.31–1.56)	1.36 (0.64–2.87)	0.41
Multivariable OR (95% CI)[c]	1.00 (reference)	1.41 (0.37–5.41)	1.31 (0.28–6.05)	0.75	1.00 (reference)	0.61 (0.25–1.62)	1.33 (0.58–3.09)	0.47
Daidzein (ng/mL)	<12.0	12.0–62.8	>62.8		<13.1	13.1–47.8	>47.8	
Number of cases/controls	12/19	11/19	5/16		22/43	15/38	25/40	
Matching variables adjusted OR (95% CI)[b]	1.00 (reference)	0.83 (0.28–2.43)	0.33 (0.07–1.58)	0.19	1.00 (reference)	0.75 (0.32–1.77)	1.32 (0.57–3.03)	0.53
Multivariable OR (95% CI)[c]	1.00 (reference)	1.21 (0.29–5.13)	0.55 (0.10–3.19)	0.50	1.00 (reference)	0.68 (0.25–1.82)	1.23 (0.47–3.21)	0.70
Glycitein (ng/mL)	Not detected	1.0–3.4	>3.4		Not detected	1.0–3.2	>3.2	
Number of cases/controls	12/26	9/14	7/14		16/39	17/42	29/40	
Matching variables adjusted OR (95% CI)[b]	1.00 (reference)	1.43 (0.47–4.31)	1.09 (0.26–4.50)	0.81	1.00 (reference)	1.02 (0.44–2.36)	1.95 (0.84–4.53)	0.10
Multivariable OR (95% CI)[c]	1.00 (reference)	2.96 (0.60–14.61)	1.97 (0.35–10.93)	0.48	1.00 (reference)	1.05 (0.41–2.68)	2.14 (0.82–5.59)	0.11
Equol (ng/mL)	Not detected	1.0–12.3	>12.3		Not detected	1.0–11.2	>11.2	
Number of cases/controls	18/20	4/16	6/18		22/46	13/35	27/40	
Matching variables adjusted OR (95% CI)[b]	1.00 (reference)	0.33 (0.10–1.14)	0.40 (0.13–1.25)	0.06	1.00 (reference)	0.76 (0.33–1.79)	1.44 (0.70–2.94)	0.33
Multivariable OR (95% CI)[c]	1.00 (reference)	0.26 (0.06–1.17)	0.44 (0.13–1.49)	0.11	1.00 (reference)	0.74 (0.27–2.00)	1.35 (0.60–3.04)	0.47

[a]Linear trends were tested using the exposure categories as ordinal variables.
[b]Matching variables were age, sex, public health center area, fasting status at blood collection, hepatitis virus infectious status, and baseline menopausal status (for women).
[c]Adjusted for alcohol consumption (never, past, or regular drinker for women; never, past, <150, 150 to <450, ≥450 g/week ethanol for men), BMI (<25.0, ≥25.0 kg/m²), diabetes (yes, no), and coffee consumption (almost never, once a week to <1 cup/day, ≥1 cup/day).

association remained essentially unchanged. We thought, therefore, that plasma concentrations of isoflavones would tend to be positively associated with the occurrence of primary liver cancer in women with hepatitis virus infection, although we suspected sufficient statistical power might not be obtained due to the relatively small sample size. In the present study, however, we observed no apparent association.

One possible explanation for this inconsistency is that plasma concentrations of isoflavones might not actually reflect dietary intake of isoflavones among participants with hepatitis virus infection. The half-lives of genistein and daidzein in the blood are reported to be 8.4 hours and 5.8 hours, respectively (15). Plasma concentrations of isoflavones reflect the intake of isoflavones in a dose-dependent manner (16, 17), and these concentrations are known to depend on the time elapsed since the last meal. Therefore, we matched fasting times in the cases and controls to minimize any exposure misclassification caused by differences in fasting times. However, plasma isoflavone concentrations are markers of short-term isoflavone exposure, whereas the results of our food-frequency questionnaire on dietary intake of isoflavones reflect personal dietary habits over long periods of time. Short-term exposure does not necessarily correlate with long-term exposure. Even so, in the general population of our validation study, isoflavone concentrations in the blood correlated reasonably well with isoflavone intake as estimated from the questionnaire (Spearman correlation coefficient = 0.33 for genistein and 0.31 for daidzein in the combined data on both sexes; ref. 18): plasma isoflavone concentrations seemed to be maintained in Japanese who like isoflavone-rich foods. Our earlier work within the JPHC Study on the associations between isoflavones and cancers in other sites supports this assumption, with similar associations observed between the results of a cohort study using a food-frequency questionnaire and those of a nested case–control study using plasma concentrations (19–26). Therefore, the present results indicate a possibility that plasma concentrations of isoflavones do not reflect dietary intake of isoflavones in people infected with hepatitis virus.

The correlation coefficients for plasma concentrations and dietary intake (as estimated from the food-frequency questionnaire) of genistein and daidzein in the present study population infected with hepatitis virus (Spearman correlation coefficient for genistein = 0.12 in women and 0.27 in men, and for diadzein = 0.09 in women and 0.29 in men) tended to be lower than those in the general population of our validation study (18). Although these findings might be the result of chance, another possible explanation lies in the metabolism of isoflavones: isoflavones are absorbed in the upper small intestine and conjugated in the liver (27); conjugated metabolites are excreted in the bile, are deconjugated in the lower bowel, and are absorbed again (27), meaning that an enterohepatic circulation is formed. In the present study, we targeted people infected with hepatitis virus. We hypothesize, therefore, that the conjugation metabolism of isoflavones in the liver is delayed when liver function is impaired by virus-related liver disease, decreasing the enterohepatic circulation volume of isoflavones in patients with virus-related liver disease, and thereby reducing isoflavone concentrations in the blood. We also hypothesize that the metabolism of isoflavones gradually declines as virus-related liver diseases progress. If this hypothesis is correct, we might observe a positive association between plasma isoflavone concentrations and liver cancer in subgroup analyses excluding participants with severe hepatitis and liver cirrhosis, or after

adjustment for liver disease stage. However, a limitation of this study is that we had no information on the clinical severity of liver disease related to HBV or HCV infection. Because of the relatively small number of cases of liver cancer, it was also difficult to find any meaningful association after excluding cases diagnosed in the first several years of follow-up. Careful consideration to this hypothesis leads us to believe that the null association we observed in women might be partially explained by the variance between plasma isoflavone concentrations and dietary isoflavone intake caused by changes in the metabolism of isoflavones related to liver disease with hepatitis virus. Further epidemiologic and experimental investigations are needed to examine the association between biomarkers of isoflavones at the early stage of virus-related liver disease and liver cancer.

As in our previous study based on dietary isoflavone intake (3), we found no association between plasma isoflavone concentrations and primary liver cancer in men. Testosterone is reportedly associated with the risk of hepatocellular carcinoma (the most common form of primary liver cancer; refs. 28, 29). However, the earlier studies did not address concerns about the association between soy isoflavones and the male sex hormone (30). We found no evidence that isoflavones play any role in the etiology of liver cancer in men, and we believe that even if they do have a role, it is small.

The major strength of our study is that it is, to our knowledge, the first prospective study to evaluate the association between plasma isoflavones and primary liver cancer. We used objective measures that reflected dietary intake of isoflavones and individual differences in absorption and metabolism (4), and attempted to elucidate the influences of exposure to isoflavones in the liver carcinogenesis. In addition, using blood samples for exposure assessment made it possible to examine the role of equol, which cannot be assessed from food-frequency questionnaires. Another strength is its nested case–control design. Blood samples were collected before cancer diagnosis, and the cases and controls were selected from the same population participating in the JPHC Study. ORs estimated in the nested case–control design represent a better approximation of risk ratios (31), allowing our study to overcome the disadvantages inherent in the case–control design. However, caution is necessary in generalizing the results, because our participants were limited to those who answered the questionnaire and provided blood samples (32). Also, primary liver cancer that was unrelated to HBV or HCV infection was not considered in this study.

In conclusion, we found no apparent association between plasma concentrations of isoflavones and the risk of primary liver cancer in participants of either sex with hepatitis virus infection. To clarify the role of isoflavones in the etiology of liver cancer, further studies using both biomarkers and data on dietary intake of isoflavones are required.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Michikawa, M. Inoue, T. Yamaji, M. Iwasaki, M. Mizokami, S. Tsugane
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Takehiro Michikawa, Manami Inoue, Norie Sawada, et al.

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