RESEARCH ARTICLE

Counts of *Slackia* sp. strain NATTS in Intestinal Flora are Correlated to Serum Concentrations of Equol both in Prostate Cancer Cases and Controls in Japanese Men

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Abstract

Background: Isoflavones, which are included in soybeans, have been suggested to protect against prostate cancer. Equol, one of isoflavones, is an intestinally derived bacterial metabolite of daidzein. A newly identified equol-producing bacterium, *Slackia* sp. strain NATTS, with a high equol-producing activity was isolated from human feces in Japanese adults. Counts of *Slackia* sp. strain NATTS in intestinal flora have not been assessed with regard to prostate cancer risk. In this study, we investigated the association of serum isoflavones and counts of *Slackia* sp. strain NATTS with prostate cancer risk in a case-control study. Materials and Methods: Concentrations of isoflavones and counts of *Slackia* sp. strain NATTS in feces were measured from 44 patients with prostate cancer and 28 hospital controls. The risk of prostate cancer was evaluated in terms of odds ratios (ORs) and 95% confidence intervals (CIs) by the logistic regression analysis. Results: The detection proportions of *Slackia* sp. strain NATTS in cases and controls were 34.1% and 25.0%, respectively. Counts of *Slackia* sp. strain NATTS were significantly correlated with serum concentrations of equol both in cases and controls (Spearman correlation coefficients, r_s=0.639 and r_s=0.572, p<0.01, respectively). Serum concentrations of genistein, daidzein, glycitein, and equol were not significantly associated with risk of prostate cancer. Conclusions: This study found that counts of *Slackia* sp. strain NATTS correlated with serum concentrations of equol both in prostate cancer cases and controls, but serum isoflavone concentrations were not associated with risk of prostate cancer in our patients.

Keywords: *Slackia* sp. strain NATTS - equol - daidzein - prostate cancer - Japanese men

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Introduction

Recently, researchers have focused on the protective effect of isoflavones, in particular, equol converted from daidzein, in the epidemiology of prostate cancer (Kurahashi et al., 2008; Park et al., 2009; Travis et al., 2012). Isoflavones are one of the phytoestrogens that are possible preventive factors in several hormone-dependent cancers such as prostate cancer and breast cancer (Zhu et al., 2011; Khan et al., 2012), cardiovascular disease (Wong et al., 2012; Usui et al., 2013), menopausal symptoms (Aso et al., 2012; Jenks et al., 2012), and osteoporosis(Scheiber et al., 2001; Tousen et al., 2011). Especially, genistein and daidzein, which are aglycones of isoflavones, were found to have potential roles in the prevention of prostate cancer (Akaza et al., 2004; Nagata et al., 2007; Gardner et al., 2009).

Generally, isoflavones have a binding affinity to estrogen β-receptors (Kuiper et al., 1998) and equol which is colonal bacterial metabolite of daidzein can bind to sex hormone-binding globulin. Equol displays the highest affinity to 5α-dihydrotestosterone, and inhibits the growth of prostate cancer cells *in vitro* and *in vivo* (Lund et al., 2011). Equol is believed to have a stronger protective effect than daidzein against development of prostate cancer (Lund et al., 2004).

In soybean foods, isoflavones exist in the aglycone (genistein, daidzein, and glycitein) and glucoside forms (Richelle et al., 2002; Setchell et al., 2002). When soybeans are taken in, glucoside forms are hydrolyzed

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into absorbable aglycones. Furthermore, daidzein is metabolized into equol by intestinal bacteria in the colon (Rowland et al., 2003). The ability to produce equol depends on the presence of certain intestinal bacteria (Rowland et al., 2003). The proportion of equol-producer varies from 30% to 60%, and is higher among Asians and the elderly (Akaza et al., 2004; Fujimoto et al., 2008). Equol-producing intestinal flora have been reported *in vitro* human fecal culture (Setchell et al., 2010; Sugiyama et al., 2013). A new equol-producing bacterium, *Slackia* sp. strain NATTS was recently isolated from a healthy human fecal culture by addition of prebiotic sorbose. *Slackia* sp. strain NATTS was found to transform daidzein into equol, and belong to genus *Slackia* family *Coriobacteriaceae* by 16S rRNA gene sequence-based analysis (Tsuji et al., 2010). Tsuji et al. (2012) showed that, in *Slackia* sp. strain NATTS, the daidzein-to-equol conversion reaction proceeded by the action of a series of three enzymes. Other several strains converting daidzein into equol have already been found by cultivation *in vitro* from human intestinal bacteria. For instance, *Enterococcus faecium* EPI1, *Finegoldia magna* EPI3 and *Lactobacillus mucosae* EPI2 (Decroos et al., 2005), *Aldercreutzia equolifaciens* (Maruo et al., 2008), *Eggerthella* sp. YY7918 (Yokoyama et al., 2008), *Slackia* sp. HE8 (Matthies et al., 2009) and *Slackia equolifaciens* strain DZE (Jin et al., 2010) were isolated and identified. In this case-control study, we examined the association of serum isoflavones, and *Slackia* sp. strain NATTS with prostate cancer risk among Japanese men.

Materials and Methods

Study population/design

This case-control study was performed from September 2011 to May 2013 at Sapporo Medical University Hospital. Informed consent was obtained from every study subject. This study was approved by ethics committee of Sapporo Medical University.

A total of 44 cases were recruited from inpatients at the Department of Urology. They were newly diagnosed with clinically localized prostate cancer, for first time no more than 1 year from the diagnosis, and all have undergone laparoscopic radical prostatectomy.

A total of 28 controls were selected from inpatients at the Department of Dermatology and Department of Cardiovascular, Renal and Metabolic Medicine of the same hospital. Disease categories were dermatosis, hypertension and cardiovascular disease in the controls. Exclusion criteria for controls had a clinical history of benign prostatic hypertrophy, other prostatic disease, malignant tumors, and a serum prostate specific antigen (PSA) concentration more than 4.0ng/ml. In addition, exclusion criteria for cases and controls were dietary restriction and having taken antibiotics within a month of the survey.

The assayed isoflavones were genistein, daidzein, glycitein and equol. Fasting blood samples were drawn before breakfast and all samples were subsequently analyzed at a single laboratory (SRL, Inc. Tokyo, Japan) under blinding in cases and controls. Serum concentrations of isoflavones in the samples were measured using triple quadrupole tandem liquid chromatography-mass spectrometry (LC-MS/MS). Controls were measured with PSA concentrations. Fresh fecal samples collected from both cases and controls were analyzed at a single laboratory at the Yakult Central Institute for Microbiological Research, Tokyo, Japan. The 16S rRNA gene fragments of *Slackia* sp. strain NATTS and related strains were amplified from the total RNA extracted from the fecal samples in order to determine the number of species by RT-qPCR (reverse transcription-quantitative PCR) (Tsuji et al., 2010). Detection limits were less than 0.5ng/ml for all isoflavones concentrations and 3.9 $\log_{10}$cell/g for counts of *Slackia* sp. strain NATTS. The questionnaire included the following information such as age, body height, body weight, and smoking habits.

Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) for the risk of prostate cancer were estimated by the median cut-off level of serum isoflavones concentration on the frequency distribution of controls using a logistic regression models. For serum equol concentration and counts of *Slackia* sp. strain NATTS, the lower categories comprised study subjects with amounts below the detection limit (<0.5 ng/ml and <3.9 $\log_{10}$cells/g, respectively). ORs and 95% CIs were adjusted with age, body mass index (BMI) and smoking status. Student's t-test, chi-squared test, Mann-Whitney U test, Fisher's exact test, and Spearman correlation coefficients were also applied. All analyses were performed using SPSS version 18.0 and a $p<0.05$ was considered statistically significant.

Results

Characteristics of prostate cancer cases and controls are shown in Table 1. Average age, average BMI, smoking status, and distributions of serum isoflavones concentration and counts of *Slackia* sp. strain NATTS were not significantly different between cases and controls.

Table 2 shows clinical stage and pathologic Gleason score in prostate cancer cases. The largest proportion of clinical stage was T1a-c as 50.0%, and the largest

Table 1. Characteristics of Prostate Cancer Cases and Controls[a]

Variables	Cases (n=44)	Controls (n=28)	p[b]
Age (years)	64.7±6.6	63.4±6.6	0.559
BMI (kg/m^2)	24.2±3.2	23.1±3.3	0.186
Smoking status			0.753
Never	7 (15.9%)	3 (10.7%)	
Former	27 (61.4%)	17 (60.7%)	
Current	10 (22.7%)	8 (28.6%)	
PSA level (ng/ml)	6.0[c] (5.3-9.3)	0.8 (0.5-1.9)	<0.001
Genistein (ng/ml)	40.0 (19.6-72.5)	59.3 (31.9-112.9)	0.067
Daidzein (ng/ml)	15.2 (3.8-36.8)	19.3 (8.6-31.6)	0.393
Glycitein (ng/ml)[d]	0.0 (0.0-1.6)	1.0 (0.0-1.9)	0.155
Equol (ng/ml)[d]	0.0 (0.0-9.5)	0.0 (0.0-10.2)	0.558
Slackia sp. strain NATTS[e] ($\log_{10}$cells/g)	0.0 (0.0-7.7)	0.0 (0.0- 4.1)	0.172

[a]Values are expressed as mean±SD or median [interquartile range]; [b]p for t-test, χ^2test or Mann-Whitney U test; [c]Preoperative value; [d]Values below the detection limit (<0.5ng/ml) were regarded as zero; [e]Values below the detection limit (<3.9$\log_{10}$cell/g) were regarded as zero

Table 2. Clinical Status of Prostate Cancer Cases

		Cases (n=44)	%
Clinical stage	T1a-c	22	50.0
	T2a	13	29.5
	T2b	4	9.1
	T2c	4	9.1
	T3a	1	2.3
Pathologic gleason score	5 or 6	5	11.4
	3+4	19	43.2
	4+3	10	22.7
	8-10	10	22.7

Table 3. Proportion of *Slackia* sp. strain NATTS and Equol above and below the Detection Limits in Cases and Controls

	Cases (n=44)		Controls (n=28)		P*
	n	%	n	%	
Slackia sp. strain NATTS ≥3.9 $\log_{10}$cells/g					1.000
(detectable subjects)	(15/44)	(34.1)	(7/28)	(25.0)	
Equol ≥0.5(ng/ml)	12	80.0	6	85.7	
Equol <0.5(ng/ml)	3	20.0	1	14.3	
Total	15	100.0	7	100.0	
Slackia sp. strain NATTS <3.9 $\log_{10}$cells/g					0.166
(non-detectable subjects)	(29/44)	(65.9)	(21/28)	(75.0)	
Equol ≥0.5(ng/ml)	4	13.8	7	33.3	
Equol <0.5(ng/ml)	25	86.2	14	66.7	
Total	29	100.0	21	100.0	

*Fisher's exact test

Table 4.Spearman's Correlation Coefficients of Age, BMI, Serum Concentrations of Isoflavones and Counts of *Slackia* sp. strain NATTS in Feces among the Cases and Controls

		Age	BMI	Genistein	Daidzein	Glycitein	Equol
BMI	Cases	-0.523**					
	Controls	-0.236					
Genistein	Cases	0.071	0.126				
	Controls	-0.040	-0.131				
Daidzein	Cases	-0.054	0.182	0.828**			
	Controls	-0.015	0.136	0.796**			
Glycitein	Cases	0.005	0.077	0.697**	0.845**		
	Controls	0.023	0.066	0.572**	0.772**		
Equol	Cases	0.036	0.097	0.216	0.221	0.259	
	Controls	-0.008	0.018	-0.031	0.068	0.317	
NATTS*	Cases	0.052	0.227	-0.097	-0.074	-0.029	0.639**
	Controls	-0.012	0.212	-0.091	0.037	0.179	0.572**

**Slackia* sp. strain NATTS; **p<0.01

Table 5.Odds Ratios (ORs) and 95% Confidence Intervals (CIs) of Prostate Cancer for Serum Isoflavones Concentration and Counts of *Slackia* sp. train NATTS in Feces with Logistic Regression Analysis

Variables[a]		Cases	Controls	OR[b] (95% CI)	p value
Genistein (ng/ml)	≤59.3	30	14	1.00	
	>59.3	14	14	0.43 (0.15-1.23)	0.114
Daidzein (ng/ml)	≤19.3	25	14	1.00	
	>19.3	19	14	0.69 (0.26-1.87)	0.465
Glycitein (ng/ml)	≤1.0	31	14	1.00	
	>1.0	13	14	0.41 (0.15-1.15)	0.090
Equol (ng/ml)	<0.5[c]	28	15	1.00	
	≥0.5	16	13	0.63 (0.23-1.70)	0.627
Slackia sp. strain NATTS ($\log_{10}$cells/g)					
	<3.9[c]	29	21	1.00	
	≥3.9	15	7	1.27 (0.42-3.83)	0.677

[a]Median distribution based on controls; [b]ORs adjusted for age, BMI and smoking; [c]Not detected

proportion of pathologic Gleason score was 3+4 as 43.2%.

As shown in Table 3, *Slackia* sp. strain NATTS in feces of cases and controls were 34.1% and 25.0%, respectively. Among detectable subjects for counts of *Slackia* sp. strain NATTS, proportions of subjects for detectable concentration of equol in cases and controls were 80.0% and 85.7%, respectively, and were not significantly different between cases and controls. Among non-detectable subjects for counts of *Slackia* sp. strain NATTS, proportions of subjects for non-detectable concentration of equol in cases and controls were 86.2% and 66.7%, respectively, and were not significantly different between cases and controls.

Table 4 present comparisons of data for the Spearman's correlation coefficients in the associations between age, BMI, serum isoflavones concentration, and *Slackia* sp. strain NATTS, in cases and controls. Counts of *Slackia* sp. strain NATTS were significantly correlated with serum equol in cases (r_s=0.639, p<0.01) and in controls (r_s=0.572, p<0.01). Age was significantly negatively associated with BMI in cases (r_s=-0.523, p<0.01), but not in controls. Serum concentrations of genistein and daidzein were significantly correlated with each other both in cases and controls (r_s=0.828 and r_s=0.796, p<0.01, respectively). Serum concentrations of genistein and glycitein were significantly correlated with each other both in cases and controls (r_s=0.697 and r_s=0.572, p<0.01, respectively). Serum concentrations of daidzein and glycitein were significantly correlated with each other both in cases and controls (r_s=0.845 and r_s=0.772, p<0.01, respectively).

Table 5 shows the ORs and 95% CIs of prostate cancer risk according to serum isoflavones concentration and counts of *Slackia* sp. strain NATTS. Serum concentrations of isoflavones were not associated with risk of prostate cancer (genistein; OR=0.43, 95% CI 0.15-1.23), (daidzein; OR=0.69, 95% CI 0.26-1.87), (glycitein; OR=0.41, 95% CI 0.15-1.15), (equol; OR=0.63, 95% CI 0.23-1.70). Similarly, counts of *Slackia* sp. strain NATTS were not associated with prostate cancer risk (OR=1.27, 95% CI 0.42-3.83).

Discussion

In this study, serum concentrations of genistein, daidzein, glycitein, and equol were not associated with risk of prostate cancer. Large prospective studies in Europe indicated that higher a plasma concentration of genistein was significantly associated with a lower risk of prostate cancer (Travis et al., 2009). However, plasma concentrations of daidzein and equol were not significantly associated with risk of prostate cancer (Travis et al., 2009). In a nested case-control study among Japanese men, high serum levels of isoflavones, especially equol, significantly tended to reduce the risk of prostate cancer (Ozasa et al., 2004). Also, in another nested case-control study, the highest levels of genistein and equol were significantly associated with a decreased risk of localized prostate cancer (Kurahashi et al., 2008). However, several previous epidemiological studies (Ward et al., 2008; Travis et al., 2012) showed that serum isoflavones concentrations were not associated with risk of prostate cancer. To

our knowledge, there are no reports which investigate associations between *Slackia* sp. strain NATTS and risk of prostate cancer.

In this study, we found that counts of *Slackia* sp. strain NATTS in feces were significantly associated with serum equol concentrations both in cases and controls. Subjects with detectable counts of *Slackia* sp. strain NATTS had detectable equol in high proportions. These results have suggested that *Slackia* sp. strain NATTS have a potent equol-producing ability from daidzein.

Tsuji et al. (2010) reported that the detection rate of *Slackia* sp. strain NATTS in the feces of 40 healthy volunteers were 33% in subjects under 40 years old and were 47% in subjects over 40 years old. In the present study, detection proportions of subjects for *Slackia* sp. strain NATTS in cases and controls were smaller than these percentages shown by Tsuji et al. (2010).

We found that correlation between serum genistein and daidzein were statistically significant, and were correlated with serum glycitein both in cases and controls. Ozasa et al. (2004) showed that serum genistein and daidzein were very strongly correlated with each other. Mori et al. (2008) reported that serum daidzein concentration in patients with prostate cancer were significantly correlated with serum concentration of genistein and glycitein. Our results were consistent with these findings in cases and controls.

Our study has also shown that serum concentration of genistein and daidzein are not related to serum equol concentration either in cases or controls. Mori et al. (2008) showed that the serum concentration of equol was not correlated with the serum concentration of daidzein, genistein or glycitein. Sawada et al. (2010) indicated that plasma genistein was not correlated with equol in Japanese men. These results were consistent with the results from our study, although Ozasa et al. (2004) showed that serum concentration of genistein and daidzein were moderately correlated with serum equol concentration in controls.

Several limitations must be considered in this study. First, the sample size of this case-control study was small, and it caused reduced statistical power to identify as a risk factor for prostate cancer. Second, blood samples were collected after the diagnosis of prostate cancer cases and controls, although any case with prostate cancer had not been treated with chemotherapy, radiation or hormonal therapy. Third, controls were recruited from inpatients, but not from the general population, and it may have induced a selection bias.

In conclusion, this study indicated that counts of *Slackia* sp. strain NATTS in feces were correlated to serum concentration of equol in Japanese men. Serum isoflavones concentrations were not associated with risk of prostate cancer. Further study is required to confirm this finding.

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