cancer is currently the most common female cancer, as in Western countries [1]. During this period, an approximately 20% reduction in the consumption of total fruits and vegetables has been reported [2].

Fruit and vegetables are rich sources of various bioactive compounds and micronutrients, such as vitamins, phytoestrogens and carotenoids [3]. These are potentially anticarcinogenic substances for which preventive effects against cancer have been proposed [4]. A majority of epidemiological evidence does not support an overall preventive association [5, 6], however, and the latest report from the World Cancer Research Fund made no conclusion due to insufficient evidence [7].

One explanation for the lack of any firm conclusion on the association of total fruits and vegetables on overall breast cancer risk might be the possible heterogeneity of impact on risk by sub-type of fruit or vegetable, or subtype of breast tumor, such as estrogens—and/or progesterone receptor (ER/PR) status. For instance, mechanisms hypothesized to explain a putative decrease in risk by cruciferous vegetables include a hormone-related mechanism [8, 9], inhibition of cell growth, and induction of apoptosis [10]. To date, the impact of consumption of total fruit and vegetables in consideration of estrogen and/or progesterone receptor (ER/PR) status has been reported by several studies [11, 12, 13, 14], as well as most recently in a large pooled analysis [15] of 20 cohort studies with the baseline data of the Japan Public Health Center-based Prospective Study (JPHC) [15]. Although this pooled overall result showed a statistically significant inverse association between total vegetable consumption and ER-negative (−) breast cancer risk, the study-specific risk estimates in JPHC at the baseline survey suggested the opposite direction to that in the overall pooled result for 20 studies [15]. However, the baseline survey of JPHC asked about information for four fruits and three vegetables and did not involve several commonly consumed vegetables in Japan, such as Japanese radish, Chinese cabbage, or Komatsuna. Further, in general, commonly consumed vegetables in Japan are not the same as those in Western countries [16]. For example, the food supply in 2009 (capita/day) was 5 kcal for tomatoes, 11 for onions, and 57 for other vegetables in Japan, versus respective values of 21, 9, and 46 kcal. Here, we prospectively evaluated the impact of total and subgroup fruit and/or vegetable intake on the risk of breast cancer in consideration of ER/PR status among 47,289 Japanese women using data from the 5-year follow-up survey of the JPHC with detailed dietary information. We also conducted secondary analyses to assess which specific vegetables contribute to the prevention of breast cancer.

Materials and methods

Study population

The study population of the JPHC study was defined as Japanese inhabitants of 11 public health center (PHC) areas and consisted of two cohorts, with Cohort I launched in 1990 and Cohort II in 1993. Details of the study design have been described previously [17]. The present study was approved by the institutional review board of the National Cancer Center, Tokyo, Japan.

A total of 140,420 subjects were invited to participate in the baseline survey (1990–1994), of whom 71,698 were women. Subjects in the Tokyo-Katsushika PHC area ($n = 4,178$) were excluded because complete information on cancer incidence were not available. Three self-reported questionnaires were sent, one each at baseline (response rate 83 %), 5-year follow-up (1995–1998; response rate 79 %) and 10-year follow-up (2000–2003; response rate 77 %).

The present analysis was conducted in 53,600 women who completed the 5-year follow-up survey questionnaire, which covered information on diet in greater detail than that at baseline. We excluded those who moved before the start of follow-up or who could not complete follow-up, as well as those with a self-reported history of cancer before the start of the follow-up ($n = 3,347$). We also excluded women with missing data on total fruit or total vegetable intake, or with extreme total energy intake (±2SD) at the 5-year follow-up survey ($n = 971$). Consequently, 47,289 women were eligible for inclusion in the present analysis.

Exposure measurement

Dietary assessment was done using a validated self-administered food frequency questionnaire (FFQ). The 5-year follow-up survey inquired about 138 food and beverage items [18, 19], including 16 fruits and 30 vegetables. We asked about the average frequency and portion size in the past year, with nine frequency categories ranging from *almost never* to *7 or more times per day*. Standard portions/units were specified for each food item, with the three amount choices of small (50 % smaller than standard), medium (same as standard), and large (50 % larger). For seasonal fruit and vegetable products, we asked about the frequency of intake when in season and calculated the consumption (g/day) by taking account of seasonal length and portion size.

The consumption of fruits and vegetables [grams per day (g/day)] as well as other estimated dietary/nutritional information was calculated with reference to the Standard Tables of Food Composition in Japan, Fifth Revised Edition [20]. Items were classified into several sub-groups,

including eight cruciferous vegetables (cabbage, Japanese radish, Chinese cabbage, Komatsuna, broccoli, leaf mustard, qing-geng-cai, and chard), five green vegetables (spinach, Chinese chives, mugwort, green pepper, and garland chrysanthemums), four carotenoid-rich yellow vegetables (carrots, pumpkins, tomatoes, and tomato juice), three tomato products (tomato, tomato juice, and ketchup) and two citrus fruits (mandarin oranges, other oranges).

Nutritional covariates, except alcohol intake, were adjusted for total caloric intake using the residual method [21].

Validity and reproducibility of the FFQ for dietary assessment has been evaluated [22, 23]. Spearman correlation coefficients between the FFQ and 14- and 28-day dietary records as the objective standard were 0.25 for fruits and 0.34 for vegetables among 113 women [22]. Reproducibility was evaluated by administering two FFQs one year apart to 108 women, and showed Spearman correlation coefficients of 0.50 for fruits and of 0.53 for vegetables (energy-adjusted) [23].

Information on other covariates, such as anthropometric and reproductive characteristics, smoking status and menopausal status, was also collected using the self-administered questionnaire at 5 years and updated in the analysis whenever reasonable information was available from the 10-year follow-up survey.

Ascertainment of breast cancer cases and follow-up of the cohort

Breast cancer cases were identified through active patient notification from major local hospitals in the study area and data linkage with population–based registries, with permission from the local governments responsible for the registries. Breast cancer cases were defined with reference to the Third Edition of the International Classification of Diseases for Oncology [24] as codes C500–509. Eight cases were identified by information on death certificates (i.e. Death Certificate Notification), of which five had no information on diagnosis (i.e. Death Certificate Only). Diagnosis was microscopically confirmed for 97 % of all cases. ER and PR status were evaluated by either immunohistochemical assay or enzyme-linked immunoassay. The cut-off point for a positive status for ER and PR was defined by clinical estimation for medical treatment or was specified by the assay method.

Subjects were entered into the study on the administration date of the 5-year follow-up survey and contributed person-time from the 5-year follow-up survey to the date of diagnosis of cancer, date of death, date of migration out of the study area, or end of follow-up (31 December 2007), whichever occurred first.

Dates of death were verified through linkage with the registration of deaths at the regional public health centers (PHCs) under the control of the Ministry of Health, Labour and Welfare. Dates of migration were verified through linkage with residential registries at the regional PHCs.

Statistical analysis

We used multivariable Cox proportional hazards regression models to estimate relative risks (RRs) and 95 % confidence intervals (CIs) with age as a time scale [25]. The proportional hazards assumptions were explored by Kaplan–Meier curves [26]. Participants were subdivided into quartiles according to consumption of fruits and vegetables. The multivariable adjusted model included age, height (<148, 148–151.9, 152–154.9, ≥155 cm), recent BMI (<18.5, 18.5–23.9, ≥24, missing), BMI at age 20 years (<20, 20–23.9, ≥24), smoking status (never, ever), leisure-time physical activity (≤3 days/month, 1–2 days/week, ≥3 days/week), age at menarche (≤13, 14, 15, ≥16 years, missing), age at first birth (nulliparous, <26, ≥26 years, missing), parity (nulliparous, 1–2, 3, ≥4 children, missing), age at menopause (premenopausal, ≤44, 45–54, ≥54 years), use of exogenous female hormones (EFH) (ever, never), alcohol consumption (past drinkers, never-drinkers, occasional drinkers, drinkers ≤150 g ethanol/week, drinkers >150 g ethanol/week), energy-adjusted intake of isoflavones (continuous), and use of vitamin C supplementation (user, non-user). We partly performed additional analyses with adjustment for energy-adjusted intake of vitamin C.

We performed an overall analysis as well as stratified analyses by menopausal status (pre or post).

p values for trend were tested by creating a continuous variable from the median value for each intake of exposure category, which was then included in the regression model. For the sub-analyses of ER/PR tumors, RRs were presented per 100-g increment in daily intake of each fruit or vegetable. All analyses were performed using the SAS Statistical Package Release 9.1 (SAS Institute, Cary, NC). All statistical tests were two-sided, and statistical significance was defined as $p < 0.05$.

Results

After an average of 10.2 years of follow-up, corresponding to 482,944 person-years, 452 breast cancer cases were newly diagnosed among 47,289 women. Information on ER and PR status was available for 224 cases (50 % of total), of which 105 were ER+ PR+ (47 % of all ER/PR known cases), 51 were ER+ PR−, and 61 were ER− PR−.

Table 1 Subject's characteristics at the study enrolment according to consumption of fruits and vegetables in the Japan Public Health Center-based Prospective Study (1995–2007)

Characteristic	Total fruits and vegetables (g/day)				Fruits (g/day)			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Mean, g/day (SD)	229(69)	389(39)	532 (48)	829 (211)	76(36)	174(25)	270(33)	500 (185)
Number of subjects	$n = 11,435$	$n = 12,012$	$n = 12,078$	$n = 11,764$	$n = 11,352$	$n = 11,986$	$n = 12,097$	$n = 11,854$
Age at baseline, year, mean (SD)	56.0 (8.0)	56.9 (8.0)	57.5(7.8)	58.4 (7.8)	56.6 (8.1)	56.8 (7.9)	57.5 (7.9)	58.0 (7.9)
Body mass index at age 20 years, kg/m^2, mean (SD)	21.5(2.7)	21.5(2.6)	21.6 (2.6)	21.6 (2.7)	21.5 (2.7)	21.6 (2.6)	21.6 (2.6)	21.6 (2.7)
Body mass index at recent age, kg/m^2, mean (SD)	23.5(3.3)	23.5(3.2)	23.5 (3.2)	23.5 (3.1)	23.7 (3.4)	23.5 (3.2)	23.4 (3.1)	23.4 (3.1)
Height, cm, mean (SD)	151 (16)	151(15)	151 (14)	151 (15)	151 (19)	151 (12)	151 (15)	151 (14)
Age at menarche, year, mean (SD)	14.7 (2.0)	14.7 (1.9)	14.7 (1.9)	14.8 (1.9)	14.9 (2.1)	14.7 (1.9)	14.6 (1.8)	14.6 (1.8)
Age at first birth, year, mean (SD)[a]	25.0 (3.6)	24.9 (3.4)	24.9 (3.4)	24.8 (3.3)	24.9 (3.6)	24.9 (3.4)	24.9 (3.4)	24.8 (3.3)
Number of children, n, mean (SD)	2.2(1.7)	2.3 (1.7)	2.3 (1.6)	2.3(1.6)	2.3 (1.9)	2.3(1.7)	2.3(1.6)	2.2 (1.6)
Age at menopause, year, mean (SD)	48.2 (4.8)	48.4 (4.5)	48.5 (4.5)	48.3 (4.8)	48.3 (4.7)	48.5 (4.5)	48.5 (4.5)	48.2 (4.8)
Use of exgenous female hormones (ever),%	13.1	12.2	12	12.6	12.7	12.5	12.4	12.3
Smoking status (ever),%	12.8	8.1	6.8	5.6	12.8	8.4	6.5	5.7
Alcohol drinking status (ever drinkers),%	42.9	38.5	35.5	32.3	41.5	38.4	36.4	32.9
Supplement of vitamin C, (ever),%	3.5	4	4.3	4	3.9	3.9	4	4
Total energy intake, mean(SD), kcal/day	1,873 (613)	1,879 (561)	1,867 (537)	1,824 (540)	1,853 (619)	1,918 (557)	1,871(541)	1,799 (528)
Intake of isoflavone, mean(SD), mg/day	38.6 (35.8)	41.9 (29.5)	44.3 (28.7)	45.7 (29.4)	40.3 (35.9)	42.5 (30.4)	44.0 (28.9)	43.6 (28.6)
Characteristic	Vegetables (g/day)				Cruciferous (g/day)			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Mean, g/day (SD)	104 (32)	180 (19)	254 (25)	427 (140)	23 (9.6)	48 (6.5)	73(8.7)	141 (67.3)
Number of subjects	$n = 11,462$	$n = 11,991$	$n = 12,054$	$n = 11,781$	$n = 11,361$	$n = 12,000$	$n = 12,099$	$n = 11,828$
Age at baseline, year, mean (SD)	56.3 (8.1)	56.7 (7.9)	57.5 (7.8)	58.4 (7.8)	56.7 (8.1)	56.5 (7.9)	57.1 (7.8)	58.4 (7.9)
Body mass index at age 20 years, kg/m^2, mean (SD)	21.5 (2.7)	21.5 (2.6)	21.6 (2.6)	21.7 (2.7)	21.6 (2.7)	21.5 (2.6)	21.5 (2.6)	21.7 (2.7)
Body mass index at recent age, kg/m^2, mean (SD)	23.4 (3.3)	23.4 (3.1)	23.5 (3.1)	23.7 (3.2)	23.5 (3.2)	23.4 (3.1)	23.5 (3.2)	23.6 (3.2)
Height, cm, mean (SD)	151 (17)	151(14)	151 (13)	151 (17)	151 (16)	151 (15)	152 (13)	151 (16)
Age at menarche, year, mean (SD)	14.6 (1.9)	14.6 (1.8)	14.7 (1.9)	14.9 (2.0)	14.7 (1.9)	14.6 (1.8)	14.6 (1.8)	14.9(2.0)
Age at first birth, year, mean (SD)[a]	24.9 (3.5)	24.9 (3.4)	24.9 (3.4)	24.8 (3.3)	24.9 (3.6)	24.9 (3.4)	24.9 (3.3)	24.8 (3.4)
Number of children, n, mean (SD)	2.1(1.6)	2.2 (1.6)	2.3 (1.6)	2.4(1.8)	2.2 (1.7)	2.2 (1.6)	2.3(1.6)	2.4 (1.8)
Age at menopause, year, mean (SD)	48.2 (4.8)	48.3 (4.6)	48.4 (4.6)	48.4 (4.6)	48.3 (4.6)	48.2 (4.7)	48.4 (4.6)	48.5 (4.6)
Use of exgenous female hormones (ever),%	13	12.2	12.6	11.9	12.7	12.8	12.4	11.9
Smoking status (ever),%	10.8	8.6	7.4	6.5	10.5	8.4	7.7	6.6
Alcohol drinking status (ever drinkers),%	41.0	39.3	36.4	32.4	40.5	39.5	37.2	32
Supplement of vitamin C, (ever),%	3.3	3.9	4.2	4.3	3.8	3.9	4.1	3.9

Table 1 continued

Characteristic	Vegetables (g/day)				Cruciferous (g/day)			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Mean, g/day (SD)	104 (32)	180 (19)	254 (25)	427 (140)	23 (9.6)	48 (6.5)	73(8.7)	141 (67.3)
Number of subjects	$n = 11{,}462$	$n = 11{,}991$	$n = 12{,}054$	$n = 11{,}781$	$n = 11{,}361$	$n = 12{,}000$	$n = 12{,}099$	$n = 11{,}828$
Total energy intake, mean(SD), kcal/day	1,876 (613)	1,899 (559)	1,859 (534)	1,808(542)	1,856(609)	1,919(577)	1,880(517)	1,786(541)
Intake of isoflavone, mean(SD), mg/day	37.9 (36.0)	41.2 (28.6)	44.0 (27.8)	47.3 (30.7)	40.0 (37.0)	40.4 (28.7)	43.3 (27.6)	46.8 (29.9)

BMI body mass index, *SD* standard deviation

[a] Based on information among women with available data

The amount of total fruit consumed ranged from a mean value of 83 g/day in the lowest quartile to 444 g/day in the highest, while the amount of total vegetable intake ranged from a mean of 111 g/day in the lowest quartile to 384 g/day in the highest. The following food items made a major contribution: mandarin oranges (15.5 %), apples (6.2 %), Japanese radish (6.0 %), 100 percent apple juice (5.4 %), onions (5.2 %), 100 percent orange juice (5.0 %), carrots (4.7 %), cabbage (3.5 %), and 100 percent tomato juice (3.2 %).

Women with high consumption of total fruits were more likely to be older, and have a low recent BMI, early menarche, low prevalence of EFH use, no tobacco use, low alcohol consumption, low total energy intake, and high isoflavone intake (Table 1). Women with high consumption of total vegetables were more likely to be older, and have a high BMI, late menarche, more children, low tobacco use, low alcohol consumption, vitamin C supplementation, low total energy intake and high isoflavone intake compared to women with low consumption. We observed a modest positive correlation between fruit intake and vegetable intake (Spearman's $r = 0.2$).

For total fruit and vegetable consumption, our results did not provide any substantial association with a decreased risk of breast cancer in this cohort [multivariable-adjusted RR $_{Q4\ vs.\ Q1}$ was 1.17 (95 % CI 0.89–1.53; $p_{trend} = 0.14$; Table 2)]. The corresponding result for total fruit intake was 1.35 (95 % CI 0.99–1.82; $p_{trend} = 0.051$) and that for total vegetable intake was 1.02 (95 % CI 0.77–1.34; $p_{trend} = 0.77$). By sub-types of fruits and vegetables, we observed no associations for cruciferous vegetables, green-leaf vegetables, yellow vegetables, or tomato products in overall analysis. In contrast, we observed a positive association between citrus fruits intake and breast cancer risk [multivariable-adjusted RR $_{Q4\ vs.\ Q1}$ was 1.27 (95 % CI 0.95–1.70; $p_{trend} = 0.044$; Table 2)]. However, the observed association was attenuated by adjustment for vitamin C [1.23(95 % CI 0.87–1.75; $p_{trend} = 0.093$; Table 2)].

In analyses stratified by menopausal status, an appreciable reduction in the risk of breast cancer associated with increased consumption among premenopausal women was seen for cruciferous vegetables only [multivariable adjusted RR $_{Q4\ vs.\ Q1} = 0.64$ (95 % CI 0.38 to 1.10; $p_{trend} = 0.046$; Table 3].

Consumption of total fruits and vegetables, total fruits, and citrus fruits was positively associated with breast cancer risk [corresponding $RRs_{for\ total\ fruits\ and\ vegetables} = 1.61$ (95 % CI 0.90–2.85; $p_{trend} = 0.034$); $_{for\ total\ fruits} = 2.32$ (95 % CI 1.23–4.38; $p_{trend} = 0.009$; $_{for\ citrus\ fruits} = 2.29$ (95 % CI 1.28–4.10; $p_{trend} = 0.0036$; Table 3)]. However, the observed positive association between citrus fruit intake and breast cancer risk was attenuated by

Table 2 Relative risks (RRs) and 95 % confidence intervals (CIs) for the association between fruits, vegetables and breast cancer risk in the Japan Public Health Center-based Prospective Study, 1995–2007

Food group	All (N = 47,289 452 cases)				p_{trend}	100 g continuous
	Ref	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)		RR (95 % CI)
Total fruit and vegetables						
Median (g/day)	Q1. 246 g/day	Q2. 393/day	Q3. 530 g/day	Q4. 764 g/day		
Number of cases	103	105	128	116		
Age-area adjusted	1.00 (ref.)	1.02 (0.78–1.34)	1.24 (0.95–1.61)	1.12 (0.85–1.46)	0.23	
Multivariable adjusted[a]	1.00 (ref.)	1.07 (0.81–1.40)	1.29 (0.99–1.68)	1.17 (0.89–1.53)	0.14	1.01 (0.97–1.05)
Multivariable adjusted[b]	1.00 (ref.)	1.07 (0.81–1.40)	1.29 (0.99–1.68)	1.17 (0.89–1.54)	0.14	1.01 (0.97–1.05)
Fruits						
Median (g/day)	Q1. 83 g/day	Q2. 176 g/day	Q3. 269 g/day	Q4. 444 g/day		
Number of cases	90	122	131	109		
Age-area adjusted	1.00 (ref.)	1.37 (1.04–1.81)	1.50 (1.14–1.98)	1.28 (0.96–1.71)	0.086	
Multivariable adjusted[a,c]	1.00 (ref.)	1.43 (1.09–1.89)	1.57 (1.19–2.08)	1.35 (0.99–1.82)	0.051	1.03 (0.98–1.08)
Multivariable adjusted[b,c]	1.00 (ref.)	1.42 (1.07–1.88)	1.54 (1.15–2.07)	1.28 (0.89–1.85)	0.11	1.02 (0.94–1.10)
Vegetables						
Median (g/day)	Q1. 111 g/day	Q2. 182 g/day	Q3. 252 g/day	Q4. 384 g/day		
Number of cases	108	123	105	116		
Age-area adjusted	1.00 (ref.)	1.12 (0.86–1.44)	0.93 (0.71–1.21)	0.99 (0.76–1.30)	0.63	
Multivariable adjusted[a,d]	1.00 (ref.)	1.13 (0.87–1.47)	0.95 (0.72–1.25)	1.02 (0.77–1.34)	0.77	0.98 (0.91–1.05)
Multivariable adjusted[b,d]	1.00 (ref.)	1.13 (0.87–1.46)	0.95 (0.72–1.25)	1.02 (0.77–1.34)	0.78	0.98 (0.91–1.05)
Cruciferous vegetables						
Median (g/day)	Q1. 25 g/day	Q2. 48 g/day	Q3. 73 g/day	Q4. 120 g/day		
Number of cases	116	130	98	108		
Age-area adjusted	1.00 (ref.)	1.10 (0.85–1.41)	0.81 (0.62–1.06)	0.88 (0.68–1.15)	0.10	
Multivariable adjusted[a,e]	1.00 (ref.)	1.11 (0.87–1.43)	0.83 (0.63–1.09)	0.91 (0.69–1.19)	0.18	0.90 (0.75–1.08)
Multivariable adjusted[b,e]	1.00 (ref.)	1.11 (0.87–1.43)	0.83 (0.63–1.09)	0.91 (0.70–1.19)	0.18	0.90 (0.75–1.08)
Green leaf vegetables						
Median (g/day)	Q1. 8.4 g/day	Q2. 17.8 g/day	Q3. 28.7 g/day	Q4.51.1 g/day		
Number of cases	99	125	106	122		
Age-area adjusted	1.00 (ref.)	1.20 (0.92–1.57)	0.98 (0.75–1.30)	1.09 (0.83–1.42)	0.94	
Multivariable adjusted[a,e]	1.00 (ref.)	1.23 (0.94–1.60)	0.99 (0.75–1.31)	1.12 (0.85–1.47)	0.82	0.92 (0.63–1.35)
Multivariable adjusted[b,e]	1.00 (ref.)	1.23 (0.94–1.60)	0.99 (0.75–1.31)	1.12 (0.85–1.47)	0.82	0.92 (0.63–1.35)
Yellow vegetables 4						
Median (g/day)	Q1. 15.1 g/day	Q2. 34.4 g/day	Q3. 59.1 g/day	Q4. 108.4 g/day		
Number of cases	100	109	121	122		
Age-area adjusted	1.00 (ref.)	1.08 (0.82–1.41)	1. 19 (0.91–1.55)	1.20 (0.92–1.56)	0.14	
Multivariable adjusted[a,d]	1.00 (ref.)	1.08 (0.83–1.43)	1.21 (0.93–1.58)	1.21 (0.92–1.58)	0.13	1.10 (0.96–1.26)
Multivariable adjusted[b,d]	1.00 (ref.)	1.09 (0.83–1.43)	1.21 (0.93–1.58)	1.21 (0.92–1.59)	0.13	1.10 (0.96–1.26)
Tomatoes products						
Median (g/day)	Q1. 1.7 g/day	Q2. 7.4 g/day	Q3. 22.7 g/day	Q4. 66.7 g/day		
Number of cases	118	101	108	125		
Age-area adjusted	1.00 (ref.)	0.86 (0.65–1.12)	0.89 (0.68–1.16)	1.03 (0.80–1.32)	0.76	
Multivariable adjusted[a,d]	1.00 (ref.)	0.84 (0.64–1.10)	0.86 (0.66–1.13)	0.99 (0.76–1.29)	0.97	1.03 (0.85–1.24)
Multivariable adjusted[b,d]	1.00 (ref.)	0.84 (0.64–1.10)	0.86 (0.66–1.13)	0.99 (0.76–1.29)	0.96	1.03 (0.85–1.24)
Citrus fruits (without orange juice)						
Median (g/day)	Q1. 11.8 g/day	Q2. 43.2 g/day	Q3. 85.0 g/day	Q4. 188.5 g/day		
Number of cases	105	108	129	110		
Age-area adjusted	1.00 (ref.)	1.06 (0.81–1.39)	1.34 (1.03–1.76)	1.23 (0.92–1.63)	0.061	

Table 2 continued

Food group	All (N = 47,289 452 cases)				p_{trend}	100 g continuous
	Ref	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)		RR (95 % CI)
Multivariable adjusted[a,c]	1.00 (ref.)	1.09 (0.83–1.44)	1.38 (1.05–1.82)	1.27 (0.95–1.70)	0.044	1.03 (0.95–1.13)
Multivariable adjusted[b,c]	1.00 (ref.)	1.09 (0.83–1.44)	1.38 (1.05–1.82)	1.27 (0.95–1.70)	0.045	1.03 (0.95–1.13)
Multivariable adjusted[a,c,e]	1.00 (ref.)	1.09 (0.82–1.82)	1.37 (1.03–1.82)	1.23 (0.87–1.75)	0.093	1.00 (0.88–1.13)
Multivariable adjusted[b,c,e]	1.00 (ref.)	1.09 (0.82–1.44)	1.37 (1.03–1.82)	1.23 (0.87–1.74)	0.094	1.00 (0.88–1.13)

[a] Multivariable Cox proportional hazards models were adjusted for age time-scales, area (10), height (<148, 148–151.9,152–154.9, ≥155 cm), recent BMI (<18.5, 18.5–23.9, ≥24, missing), BMI at age 20 years (<20, 20–23.9, ≥24), age at menarche (≤13, 14, 15, ≥16 years or missing), age at first birth (nulliparous, <26, ≥26 years, or missing), parity (nulliparous, 1–2, 3, ≥4), menopausal status (premenopause, age at menopause ≤44, 45–54, ≥55 years), use of exogenous female hormones (never, ever), smoking status (never, ever), leisure-time physical activity (≤3 days/month, 1–2 days/week, ≥3 days/week), alcohol intake (past drinker, never-drinker, occasional drinker, regular drinker ≤150, or >150 g of ethanol per week), total energy-adjusted intake of isoflavones, and vitamin C supplement (use or non-use)

[b] The same as model[a] with adjustment for combination variables recent BMI and menopausal status (pre or postmenopause)

[c] Additionally included total energy-adjusted intake of total vegetables (continuous)

[d] Additionally included total energy-adjusted intake of total fruits (continuous)

[e] Additionally included total energy-adjusted intake of vitamin C (continuous)

adjustment for vitamin C intake. Among postmenopausal women, we did not observe any association between any fruit or vegetable intake and breast cancer risk.

In sub-analyses which considered the joint ER and PR status of breast tumors, intake of cruciferous vegetables showed a marginal inverse association with the development of ER+ PR+ tumors [age-area-adjusted $RR_{per\ 100\ g\ increment} = 0.64$ (95 % CI 0.41–1.00; $p = 0.051$ (Table 4)], while intake of citrus fruits was positively associated with ER+ PR− tumors [$RR_{per\ 100\ g\ increment} = 1.20$ (95 % CI 1.01–1.42); Table 4). Total fruits, total vegetables, and all other subgroups of fruits and/or vegetables showed no association with a decreased risk of any ER/PR tumor subtype.

Additional analyses for specific cruciferous vegetables showed that Japanese radish contributed to the inverse association among premenopausal women [age-area adjusted $RR_{Q4\ vs.\ Q1}$ was 0.50 (95 % CI 0.29–0.84; $p_{trend} = 0.003$; text only)]. The corresponding results by ER/PR tumor subtype were 0.28 (95 % CI 0.09–0.89; $p_{trend} = 0.02$ for ER+ PR+ (28 cases) and 0.48 (95 % CI 0.09–2.71; $p_{trend} = 0.15$ for ER− PR− (14 cases); text only).

Discussion

In this large prospective cohort study among Japanese women, our overall results suggested no substantial decrease in the risk of breast cancer with increased consumption of total fruits and vegetables. Our results did suggest a positive association of total fruit intake and citrus fruit intake with increased risk of breast cancer, particularly among premenopausal women, but these results were attenuated by adjustment for vitamin C intake. Further, we also observed a substantial preventive impact of cruciferous vegetables against breast cancer among premenopausal women, but no association between either total or any subgroup of fruit and vegetable intake and breast cancer risk among postmenopausal women. With regard to ER/PR breast tumors, intake of cruciferous vegetables, in particular Japanese radish, was substantially associated with a decreased risk of ER+ PR+ tumors. Our results also suggested a significant positive association between citrus fruit intake and the risk of ER+ PR− tumors. In contrast, no association with a decreased risk of any ER/PR tumor subtype was seen for total fruits, total vegetables, or any other subgroup of fruits and/or vegetables.

Similar to our present results, previous pooled analyses have reported an overall null association for total fruit and vegetable consumption [5, 6]. Median total fruit consumption (220 g/day) in the present study was within the range of median intake in one pooled analysis (164–355 g/day) [5], while median total vegetable intake (213 g/day) was within the range of median total vegetable intake (from 77 to 262 g/day) [5].

To our knowledge, this study is the first large prospective study to investigate the association between the intake of fruits and/or vegetables and breast cancer risk with consideration of ER/PR subtype in a Japanese population alone.

Unlike our present results, total vegetable intake was inversely associated with the risk of ER-negative (−) breast cancer risk in the pooled study [15]. The pooled study did evaluate the intake of total or cruciferous vegetables and involved baseline data in the JPHC. However, this baseline data took into account only three vegetables

Table 3 Relative risks (RRs) and 95 % confidence intervals (CIs) for the association between fruits, vegetables and breast cancer risk with stratified by menopausal statu in the Japan Public Health Center-based Prospective Study, 1995–2007

Consumption	Premenopausal (n = 10,527 115 cases)					Postmenopausal (n = 36,762 337 cases)				
Food group	Ref	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)	p_{trend}	Ref	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)	p_{trend}
Total fruit and vegetables										
Median (g/day)	Q1. 223 g/day	Q2. 354 g/day	Q3. 482 g/day	Q4. 703 g/day		Q1. 255 g/day	Q2. 406 g/day	Q3. 544 g/day	Q4. 780 g/day	
Number of cases	21	23	39	32		80	74	93	90	
Age-area adjusted	1.00 (ref.)	1.11 (0.61–2.01)	1.82 (1.07–3.11)	1.43 (0.82–2.51)	0.0798	1.00 (ref.)	0.92 (0.67–1.27)	1.15 (0.85–1.56)	1.11 (0.82–1.51)	0.29
Multivariable adjusted[a]	1.00 (ref.)	1.19 (0.65–2.17)	2.05 (1.19–3.53)	1.61 (0.90–2.85)	0.034	1.00 (ref.)	0.96 (0.70–1.32)	1.19 (0.87–1.61)	1.14 (0.84–1.56)	0.23
Fruits										
Median (g/day)	Q1. 72 g/day	Q2. 160 g/day	Q3. 244 g/day	Q4. 412 g/day		Q1. 87 g/day	Q2. 182 g/day	Q3. 275 g/day	Q3. 275 g/day	
Number of cases	17	31	36	31		72	92	93	80	
Age-area adjusted	1.00 (ref.)	1.83 (1.01–3.33)	2.25 (1.25–4.04)	2.03 (1.11–3.72)	0.02	1.00 (ref.)	1.29 (0.94–1.76)	1.31 (0.95–1.80)	1.31 (0.95–1.80)	0.47
Multivariable adjusted[a,b]	1.00 (ref.)	2.02 (1.10–3.71)	2.57 (1.40–4.69)	2.32 (1.23–4.38)	0.009	1.00 (ref.)	1.34 (0.98–1.83)	1.35 (0.98–1.87)	1.19 (0.84–1.67)	0.38
Multivariable adjusted[a,b,d]	1.00 (ref.)	1.88 (1.02–3.47)	2.21 (1.18–4.13)	1.65 (0.78–3.51)	0.16	1.00 (ref.)	1.35 (0.98–1.85)	1.38 (0.98–1.93)	1.23 (0.81–1.86)	0.29
Vegetables										
Median (g/day)	Q1. 104 g	Q2. 166 g	Q3. 228 g	Q4. 348 g		Q1. 114 g	Q2. 187 g	Q3. 259 g	Q4. 393 g	
Cases	25	31	30	29		82	88	80	87	
Age-area adjusted	1.00 (ref.)	1.25(0.74–2.12)	1.15 (0.67–1.96)	0.95 (0.55–1.65)	0.75	1.00 (ref.)	1.04 (0.77–1.41)	0.93 (0.68–1.27)	0.99 (0.73–1.35)	0.79
Multivariable adjusted[a,c]	1.00 (ref.)	1.24 (0.73–2.13)	1.16 (0.67–2.01)	0.95 (0.54–1.69)	0.77	1.00 (ref.)	1.06 (0.78–1.43)	0.95 (0.70–1.30)	1.03 (0.75–1.41)	0.95
Cruciferous vegetables										
Median (g/day)	Q1. 25 g	Q2. 45 g	Q3. 66 g	Q4. 108 g		Q1. 25 g	Q2. 50 g	Q3. 75 g	Q4. 124 g	
Number of cases	34	33	23	25		80	95	77	85	
Age-area adjusted	1.00 (ref)	0.98 (0.61–1.59)	0.64 (0.38–1.09)	0.64 (0.38–1.08)	0.038	1.00 (ref.)	1.16 (0.86–1.57)	0.92 (0.67–1.26)	1.02 (0.75–1.38)	0.69
Multivariable adjusted[a,c]	1.00 (ref.)	0.99 (0.61–1.61)	0.65 (0.38–1.11)	0.64 (0.38–1.10)	0.046	1.00 (ref.)	1.19 (0.89–1.61)	0.95 (0.69–1.30)	1.06 (0.78–1.45)	0.91
Green leaf vegetables										
Median (g/day)	Q1. 8.4 g	Q2. 16.8 g	Q3. 26.8 g	Q4.46.9 g		Q1. 8.4 g	Q2. 18.0 g	Q3. 29.4 g	Q4.52.2 g	
Number of cases	29	30	30	26		70	93	80	94	
Age-area adjusted	1.00 (ref.)	0.96 (0.57–1.61)	0.94 (0.56–1.58)	0.76 (0.45–1.31)	0.33	1.00 (ref.)	1.27 (0.93–1.73)	1.05 (0.76–1.45)	1.19 (0.87–1.63)	0.56
Multivariable adjusted[a,c]	1.00 (ref.)	0.97 (0.95–1.63)	0.95 (0.57–1.60)	0.78 (0.45–1.34)	0.38	1.00 (ref.)	1.28 (0.94–1.75)	1.06 (0.77–1.47)	1.22 (0.89–1.68)	0.47
Yellow vegetables										
Median (g/day)	Q1. 14.3 g	Q2. 30.7 g	Q3. 51.8 g	Q4. 95.7 g		Q1. 15.3 g	Q2. 35.6 g	Q3. 61.2 g	Q4. 111.8 g	
Number of cases	23	28	30	34		76	82	90	89	
Age-area adjusted	1.00 (ref.)	1.22 (0.70–2.12)	1.26 (0.73–2.17)	1.35 (0.79–2.31)	0.29	1.00 (ref.)	1.07 (0.78–1.46)	1.18 (0.87–1.60)	1.15 (0.85–1.57)	0.29
Multivariable adjusted[a,c]	1.00 (ref.)	1.21 (0.69–2.22)	1.27 (0.73–2.22)	1.38 (0.79–2.40)	0.27	1.00 (ref.)	1.07(0.78–1.46)	1.18 (0.87–1.61)	1.15 (0.83–1.57)	0.32
Tomatoes products										
Median (g/day)	Q1. 2.0 g	Q2. 6.9 g	Q3. 19.0 g	Q4. 58.2 g		Q1. 1.6 g	Q2. 7.5 g	Q3. 24.0 g	Q4. 69.2 g	
Number of cases	31	23	28	33		87	73	91	86	
Age-area adjusted	1.00 (ref.)	0.78 (0.45–1.35)	0.90 (0.53–1.52)	0.98 (0.59–1.61)	0.94	1.00 (ref.)	0.83 (0.61–1.13)	1.02 (0.76–1.38)	0.96 (0.71–1.29)	0.9
Multivariable adjusted[a,c]	1.00 (ref.)	0.78(0.45–1.35)	0.87(0.51–1.49)	0.97 (0.58–1.64)	0.97	1.00 (ref.)	0.81 (0.59–1.11)	0.99 (0.73–1.34)	0.91 (0.67–1.24)	0.87
Citrus fruits (without orange juice)										

Table 3 continued

Consumption	Premenopausal (n = 10,527 115 cases)					Postmenopausal (n = 36,762 337 cases)				
Food group	Ref	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)	p_{trend}	Ref	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)	p_{trend}
Median (g/day)	Q1. 9.8 g	Q2. 37.2 g	Q3. 74.8 g	Q4. 164.9 g		Q1. 12.5 g	Q2. 45.2 g	Q3. 88.1 g	Q4. 194.4 g	
Number of cases	23	25	29	38		81	80	101	75	
Age-area adjusted	1.00 (ref.)	1.19 (0.67–2.11)	1.37 (0.78–2.41)	2.01 (1.16–3.47)	0.010	1.00 (ref.)	1.00 (0.73–1.37)	1.35 (0.99–1.84)	1.06 (0.76–1.48)	0.35
Multivariable adjusted[a,b]	1.00 (ref.)	1.29 (0.72–2.32)	1.58 (0.88–2.84)	2.29 (1.28–4.10)	0.0036	1.00 (ref.)	1.02 (0.74–1.40)	1.38 (1.01–1.88)	1.08 (0.77–1.52)	0.30
Multivariable adjusted[a,b,d]	1.00 (ref.)	1.23 (0.68–2.23)	1.45 (0.79–2.65)	1.87 (0.95–3.69)	0.0645	1.00 (ref.)	1.02 (0.74–1.41)	1.40 (1.01–1.93)	1.12 (0.74–1.67)	0.23

[a] Multivariable Cox proportional hazards models were adjusted for age time-scales, area (10), height (<148, 148–151.9,152–154.9, ≥155 cm), recent BMI (<18.5, 18.5–23.9, ≥24, missing), BMI at age 20 years (<20, 20–23.9, ≥24), age at menarche (≤13, 14, 15, ≥16 years or missing), age at first birth (nulliparous, < 26, ≥26 years, or missing), parity (nulliparous, 1–2, 3, ≥4), menopausal status (premenopause, age at menopause ≤44, 45–54, ≥55 years), use of exogenous female hormones (never, ever), smoking status (never, ever), leisure-time physical activity (≤3 days/month, 1–2 days/week, ≥3 days/week), alcohol intake (past drinker, never-drinker, occasional drinker, regular drinker ≤150, or >150 g of ethanol per week), total energy-adjusted intake of isoflavones (continuous), and vitamin C supplement (use or non-use)

[b] Additionally included total energy-adjusted intake of vegetables (continuous)

[c] Additionally included total energy-adjusted intake of fruits (continuous)

[d] Additionally included total energy-adjusted intake of vitamin C (continuous)

and the study-specific risk estimates suggested the opposite direction against the pooled result for the 20 studies [15]. Further, the observed favorable impact we saw against ER+ PR+ tumors was attributable to the intake of cruciferous vegetables, in particular Japanese radish, which was not taken into account in the pooled study. Further, consistent with previous results [27], our results for a substantial preventive impact of cruciferous vegetables against overall and ER+ PR+ breast cancer was confined to premenopausal women, who had a higher level of endogenous female hormones than postmenopausal women. Cruciferous vegetables contain glucosinolates, which are hydrolysed to isothiocyanates and indoles. Experimental and epidemiological studies suggest their potential to decrease breast cancer risk by inhibiting cell growth, inducing apoptosis [10] and affecting hormonal level through the hormone- and/or ER-related mechanism [28–30]. Our present results for ER+ PR+ tumors might support this hormone-related mechanism. Further epidemiological and experimental studies should be conducted in consideration of ER/PR status.

With regard to citrus fruits intake, we unexpectedly observed a positive association between citrus fruit intake and risk of overall and ER+ PR− tumors. Previous studies had reported inverse associations for ER− [11], for ER+ [13], and for both ER+ PR+ and ER+ PR− [12]. The results of sub-analyses for ER/PR tumors might be due to chance, because of the relatively small number of cases. For the overall results, however, a recent systematic review of five case–control studies [31] and a case–control study, the Shanghai Breast Cancer Study [32], also reported an inverse association between citrus fruits intake and breast cancer risk. Unlike these studies, our results showed a positive association overall. The quantity of citrus fruits intake in the highest quartile (Q4) was 188.5 g/day (median) in our study, while that in the highest quintile (Q5) of the Shanghai study was 30.4 g/day. Our data suggest that citrus fruits intake is correlated with vitamin C intake and that women taking supplemental vitamin C had a substantially higher risk than women who did not. Given these results, the association between citrus fruits intake and breast cancer risk might be explained by a U- or J- shaped relationship. That is, the appropriate amount of citrus fruits intake leads to risk reduction, while an extremely high intake might lead to an increased risk of breast cancer due to the extremely high dose of vitamin C. The dose-response impact of vitamin C on the development of breast cancer requires further research.

The strength of our study is its large sample size and prospective population-based cohort design. Exposure information was collected before diagnosis, ruling out the possibility of differential recall bias. Misclassification of disease was also unlikely due to the low percentages of

Table 4 Age-area adjusted relative risks (RRs) and 95 % confidence intervals (CIs) for the association between fruits, vegetables and ER/PR defined breast cancer risk in the Japan Public Health Center-based Prospective Study, 1995–2007

Variable	All ($n = 47,289$)				
ER/PR subtypes	All	ER+ PR+	ER+ PR−	ER− PR−	ERPR unknown
Number of cases	452	105	51	61	228
Food group	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)	RR (95 % CI)
Total fruit and vegetables	1.01 (0.97–1.04)	0.99 (0.91–1.07)	1.07 (0.97–1.19)	0.99 (0.89–1.10)	0.99 (0.94–1.05)
Fruits	1.03 (0.98–1.08)	1.04 (0.94–1.15)	1.12 (0.99–1.26)	1.02 (0.88–1.18)	0.99 (0.91–1.06)
Vegetables	0.97 (0.91–1.04)	0.89 (0.77–1.04)	1.04 (0.87–1.25)	0.93 (0.76–1.13)	1.00 (0.91–1.10)
Cruciferous vegetables	0.88 (0.73–1.06)	0.64 (0.41–1.00)	0.86 (0.49–1.50)	0.88 (0.53–1.47)	0.97 (0.77–1.24)
Green leaf vegetables	0.90 (0.61–1.31)	0.70 (0.30–1.61)	1.56 (0.67–3.65)	0.79 (0.27–2.31)	0.89 (0.52–1.54)
Yellow vegetables	1.11 (0.97–1.27)	1.11 (0.85–1.44)	1.22 (0.95–1.58)	1.12 (0.78–1.59)	1.08 (0.87–1.33)
Tomatoes products	1.05 (0.88–1.26)	0.62 (0.34–1.12)	1.15 (0.84–1.58)	1.07 (0.69–1.68)	1.15 (0.95–1.39)
Citrus fruits (without orange juice)	1.04 (0.95–1.13)	0.98 (0.79–1.20)	1.20 (1.01–1.42)	1.01 (0.76–1.33)	0.99 (0.87–1.12)

Unit (100 g/day)

[a] Cox proportional hazards models were adjusted for age time-scales and area (10). Multivariable adjusted model was not applied due to the small number of cases by ER/PR tumors subtype

DCN and DCO. Given the reasonable validity of fruit and vegetable intake, as measured by Spearman correlation coefficients between the FFQ and DR [33], as well as the consistency of the null results in the present and previous studies, the overall null association for total fruits and vegetables might be factual.

Several limitations should also be noted. One limitation is the small number of cases after stratification by tumor subtype and/or menopausal status. The lack of statistical power should be considered when interpreting the results. Dietary and other epidemiological information were assessed based on self-reported questionnaires, and measurement error was accordingly unavoidable. Although the FFQ-based fruits/vegetables intake was calculated using a purpose-developed database [34], some degree of seasonal, geographical and cultivational variation in micronutrient contents in food products might not be completely ruled out [35]. Our observed null association might be attributable to non-differential misclassification of exposure in prospective study design. The possibility of uncontrolled confounding and statistical chance finding due to multiple testing should also be considered.

In summary, our results do not support that the risk of breast cancer is decreased with increased consumption of total or any other subgroup of fruits and vegetables. For premenopausal women, however, our results suggest a substantial preventive association between cruciferous vegetable intake and breast cancer risk.

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Conflict of interest The authors declare no conflict of interest related to this manuscript.

Appendix

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Chemopreventive effects of 13α,14α-epoxy-3β-methoxyserratan-21β-ol (PJJ-34), a serratane-type triterpenoid, in a rat multi-organ carcinogenesis bioassay

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ABSTRACT

A novel serratane-type triterpenoid, 13α,14α-epoxy-3β-methoxyserratan-21β-ol (PJJ-34) derived from cuticles of *Picea jezoensis* Carr. var. *jezoensis*, has proved to be highly effective at suppressing carcinogenesis both in vitro and in vivo. To investigate possible anti-carcinogenic efficacy at the whole-body level, male Fischer 344 rats were subjected to an established rat multi-organ carcinogenesis bioassay (DMBDD model). After initiation with five carcinogens, groups 1–3 (20 in each) were intragastrically (i.g.) administered PJJ-34 dissolved in 1 ml of 0.5% CMC (5 times/week) at doses of 0, 5 and 10 mg/kg body weight (b.w.), respectively, until the end of week 30. PJJ-34 did not show apparent toxicity. Incidences of adenomas (100 → 75%) and carcinomas (63 → 30%) in the lung were significantly decreased in the 5 mg/kg b.w. group, and multiplicity of alveolar hyperplasias and total lung tumors (adenomas + carcinomas) were significantly reduced by both 5 and 10 mg/kg. The incidence of colorectal tumors was also significantly decreased in the 10 mg/kg group (63 → 28%) along with the multiplicity. Rat liver pre-neoplastic lesions, glutathione *S*-transferase placental form (GST-P) foci, and tumor development in the other organs were not affected. Immunohistochemical indices for proliferating cell nuclear antigen (PCNA) and cyclin D1 in normal alveolar epithelium of the lung were significantly suppressed at both doses. In conclusion, PJJ-34 is chemopreventive against lung and colon carcinogenesis without exerting apparent toxicity, and suppression of cell proliferation could play a key role in the underlying mechanisms.

1. Introduction

More than 24,000 chemicals are registered for use in Japan and humans are actually exposed to thousands of environmental chemicals with great diversity of pharmacological function and structure, simultaneously and/or sequentially during manufacture, distribution, use and disposal, or when they become pollutants in the air, water, or soil [1]. The accepted major factors for human carcinogenesis are smoking, infection, inflammation, poor nutrition

Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxy-butyl)nitrosamine; DEN, diethylnitrosamine; DHPN, 2,2′-dihydroxy-di-*N*-propylnitrosamine; DMH, 1,2-dimethylhydrazine dihydrochloride; GST-P, glutathione *S*-transferase placental form; MNU, *N*-methyl-*N*-nitrosourea; PCNA, proliferating cell nuclear antigen; PJJ-34, 13α,14α-epoxy-3β-methoxyserratan-21β-ol.

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and dietary carcinogens [2]. It is impossible to avoid all the risk factors in our environment and since cancer is now the leading cause of death in most countries, primary cancer prevention has naturally attracted attention. It has been argued that at least 50% of all cancers could be avoided by applying the existing etiologic knowledge [3]. Chemoprevention is one approach to reducing the burden of cancer and is recognized as both a clinical and basic science which is developing with incorporation of new in vitro and in vivo assays [4].

13α,14α-Epoxy-3β-methoxyserratan-21β-ol (PJJ-34), a newly-identified naturally-occurring serratane-type triterpenoid, is a promising chemopreventive agent which has proved to be highly effective for suppression of carcinogenesis both in vitro and in vivo [5]. More than ten years of our efforts to discover chemopreventive agents have been focused on serratane-type triterpenoids extracted from the highly-developed cuticles of *Picea jezoensis* (Sieb. et Zucc.) Carr. var. *jezoensis* (Pinaceae; Japanese name: Ezomatsu) and from the stem bark of *P. jezoensis* (Sieb. et Zucc.) Carr. var. *hondoensis* (Mayr.) Rehder. Earlier papers documented detailed procedures for purification of these compounds and determination of chemical structures [6–8], including isolation of PJJ-34 [9]. Methodology for discovery of chemopreventive effects of these serratane-type triterpenoids was routinely consisted of in vitro and in vivo carcinogenesis assays: first, test compounds were studied for their inhibitory effects (Trypan-Blue assay) on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells (EBV genome-carrying lymphoblastoid cells derived from a Burkitts lymphoma), and those showing more potent inhibitory potential than oleanolic acid (a well-established chemopreventive triterpenoid) were further evaluated for anti-tumorigenic effects on a two-stage mouse skin carcinogenesis model featuring 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and TPA as a promoter [5,10–14].

The medium-term rat multi-organ carcinogenesis bioassay system, generally known as the DMBDD model [15,16], features partly concomitant or partly sequential application of five genotoxic carcinogens, diethylnitrosamine (DEN), *N*-methyl-*N*-nitrosourea (MNU), *N*-butyl-*N*-(4-hydroxy-butyl)nitrosamine (BBN), 2,2′-dihydroxy-di-*N*-propylnitrosamine (DHPN), and 1,2-dimethylhydrazine dihydrochloride (DMH), with further modification of the model to obtain good tumor yields within 30 weeks [17]. Each carcinogen has different organ specificity and the DMBDD model allows induction of a variety of pre-neoplastic and neoplastic lesions at the whole-body level. The main targeted organs are the lung, colon, bladder, kidney, thyroid and liver. From accumulated experimental evidence, the DMBDD bioassay is very useful for investigation of the carcinogenic modifying potentials of various chemicals in the post-initiation phase, using neoplastic and/or established pre-neoplastic lesions as the endpoints [18].

In the present study, modifying effects of PJJ-34 were evaluated for the first time in the DMBDD model, the results providing some novel evidence in vivo.

2. Materials and methods

2.1. Animals, diet and chemical carcinogens

Male Fischer 344 rats were obtained at 5 weeks of age (Charles River Japan, Atsugi, Japan), housed four in plastic cages, and given CE-2 common basal diet (Clea Japan, Tokyo, Japan) and water ad libitum. They were kept in an environmentally controlled room maintained at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 5%, with a 12-h light/dark cycle. Body weights, food consumption and water intake were measured weekly during the experimental period. After a 1-week acclimation period, the animals were used in this study. DEN, BBN and DMH were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). MNU was from Wako Pure Chemical Industries (Osaka, Japan). DHPN was from Nacalai Tesque (Kyoto, Japan). DEN, MNU and DMH were dissolved in 0.9% physiological saline and used for intraperitoneal (i.p.) or subcutaneous (s.c.) injections. BBN and DHPN were administered to rats in their drinking water.

2.2. Extraction and synthesis of PJJ-34 from 3β-methoxyserrat-14-en-21β-ol (PJJ-1)

PJJ-34 (molecular formula: $C_{31}H_{52}O_3$) was isolated from the air-dried chopped cuticle of one *P. jezoensis* Carr. var. *jezoensis* tree (estimated over 300 years old) which growing at 1000 m in mountains around Hidaka town, Hokkaido, Japan, and the procedure with chemical structures was well documented [5,9]. The most abundant triterpene, 3β-methoxyserrat-14-en-21β-ol (PJJ-1) (1) was first obtained, and synthetic PJJ-34 was used in this study (Fig. 1a). In brief, PJJ-1 (30 g) was acetylated (Ac_2O/pyridine 1:1, 100 ml) to give 3β-methoxyserrat-14-en-21β-yl acetate (PJJ-1 acetate: 27.6 g) (2). A mixture of glacial AcOH (240 ml) and *c*-H_2SO4 (90 ml) was gradually added into (2) and was kept at room temperature for 24 h. Then, the mixture was poured into ice water and the resulting precipitate was extracted with $CHCl_3$. The extract was neutralized with 5% NaOH, washed with H_2O and dried over Na_2SO_4. Evaporation of $CHCl_3$ yielded a crystalline mass (26.7 g), which was subjected to SiO_2 column chromatography to afford 3β-methoxyserrat-13-en-21β-yl acetate (PJJ-1–13-en Ac: 20.1 g) (3). Treatment of compound (3) in boiling 0.3 N KOH/MeOH (600 ml) for 8 h and subsequent workup as usual furnished 3β-methoxyserrat-13-en-21β-ol (PJJ-1-13-en: 18.3 g) (4), followed by adding a solution of *m*-chloroperbenzoic acid (*m*-CPBA) in dry $CHCl_3$. After 24 h, the reaction mixture was washed and evaporated under reduced pressure, then the residue was purified by MPLC (240–400 mesh SiO_2) eluting with *n*-hexane-AcOEt (10: 1) to afford 13α,14α-epoxy-3β-methoxyserratan-21β-ol (PJJ-34: 15.8 g) (5) and 13β,14β-epoxy-3β-methoxyserratan-21β-ol (PJJ-43: 0.9 g) (6). Synthetic PJJ-34 (99% in purity) was identified by direct comparison with authentic natural PJJ-34, which was stored at 4 °C until use.

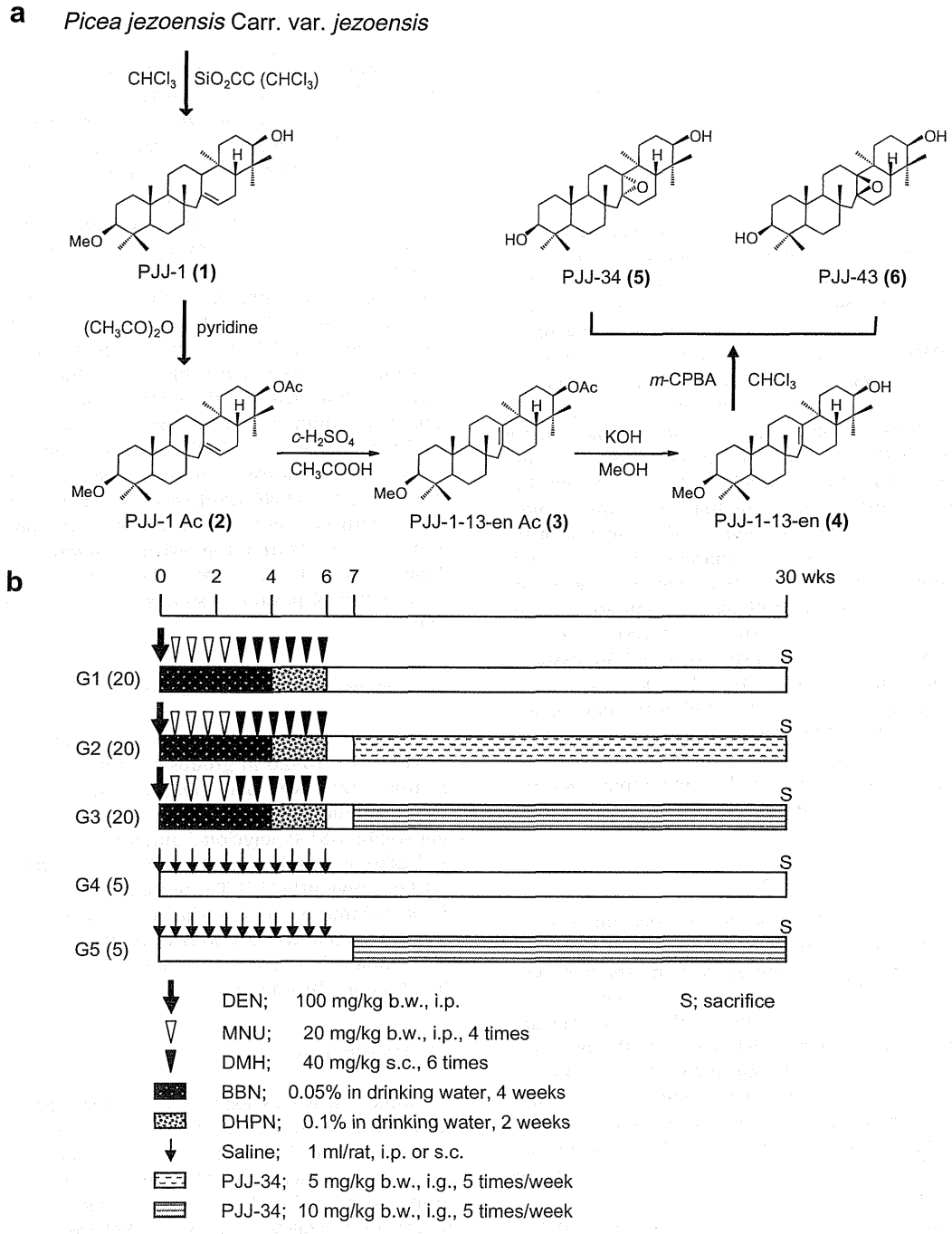

Fig. 1. Extraction and biosynthesis of PJJ-34, and experimental protocol of the DMBDD model used in this study. (a) PJJ-34 and analogues were extracted from the cuticles of a *Picea jezoensis* Carr. var. *jezoensis* tree [Ref. 5,9], which can also be synthesized from PJJ-1 and synthetic PJJ-34 (purity >99%).was used in this study. Compound (1) 3β-methoxyserrat-14-en-21β-ol; (2) 3β-methoxyserrat-14-en-21β-yl acetate; (3) 3β-methoxyserrat-13-en-21β-yl acetate; (4) 3β-methoxyserrat-13-en-21β-ol; (5) 13α,14α-epoxy-3β-methoxyserratan-21β-ol; and (6) 13β,14β-epoxy-3β-methoxyserratan-21β-ol. (b) The established rat multi-organ carcinogenesis bioassay (DMBDD model) was carried out with a modification as well as shown in a previous report [17].

2.3. Preliminary determination of the experimental doses of PJJ-34

To determine the experimental doses, 6-week-old, male F344 rats were preliminarily tested. Each time of dosing, PJJ-34 was dissolved in 0.5% CMC-Na (carboxymethyl cellulose sodium salt; Wako Pure Chemical Industries) at doses of 0, 1, 5, 10, and 20 mg/kg body weight (b.w.) for intragastric (i.g.) administration to animals 5 times per week for 4 weeks. As a result, some toxicological findings

were observed in the 20 mg/kg b.w. group, while 1, 5 and 10 mg/kg b.w. groups did not show any apparent toxicity (data not shown). Therefore, we decided to use 5 and 10 mg/kg b.w. as appropriate experimental doses in the present case.

2.4. Experimental protocol

The experimental design, which was approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School, is shown in Fig. 1b. A total of 70, 6-week-old, male F344 rats were divided into five groups. Animals in groups 1–3 (20 rats in each) underwent the modified DMBDD regimen [17], consisting of a single injection of DEN (100 mg/kg b.w., i.p.) at the commencement of experiment, followed by MNU (20 mg/kg b.w., i.p.) for 4 times and DMH (40 mg/kg b.w., s.c.) for 6 times. At the same period, 0.05% BBN in drinking water was administered during experimental weeks 1–4, followed by 0.1% DHPN during weeks 5 and 6. Animals in groups 4 and 5 (5 rats in each) received the vehicle saline instead of the carcinogen injections. After the initiation period, all rats were maintained without any treatment for 1 week for recovery and for distinction of the post-initiation period from the initiation term. Then rats in DMBDD-treated groups 1–3 received i.g. administration of PJJ-34 dissolved in 0.5% CMC at doses of 0, 5 and 10 mg/kg b.w., respectively, 5 times per week until the end of the experiment. Animals in the non-DMBDD groups 4 and 5 were given PJJ-34 in 0.5% CMC at doses of 0 or 10 mg/kg b.w. (5 times/week, i.g.), respectively, and the experiment was terminated at the end of week 30.

2.5. Organ and tissue processing

At the time of sacrifice, rats were killed under ether anesthesia and any macroscopical abnormalities were recorded. The major organs were excised and the liver, kidneys and spleen were immediately weighed. The alimentary tract from the esophagus to the rectum, the urinary bladder, and lungs with the trachea and thyroid gland were inflated gently with 10% phosphate-buffered formalin solution. The colon was cut open along the longitudinal axis and extended flat between two sheets of papers. Several skin tumors as well as other visible lesions were also sampled. All these organs and tissues were fixed in the same formalin solution.

Approximately 3 mm-thick slices were prepared for histological examination from all organs taken. Single liver slices were made from the left, intermediate and caudate lobes. Lungs were divided into 5 different portions (the left lobe and 4 right sublobes) and slices were taken from each. Three slices were routinely cut from the colon with rectum as well as visible tumors. The Bladders were routinely cut into 8 slices. All of the formalin-fixed, paraffin-embedded tissues were routinely prepared for 3 μm-thick sections and stained with hematoxylin and eosin (HE). Incidence (%) and/or multiplicity data for neoplastic and pre-neoplastic lesions in the major target organs were evaluated in this study.

2.6. Immunohistochemistry for PCNA and Cyclin D1

In order to evaluate cell proliferation, sections from the lung and colon ($n = 18–20$ in groups 1–3) were immunohistochemically stained with proliferating cell nuclear antigen (PCNA), and cyclin D1 staining was also performed (lung), as shown previously [19]. Vectastain ABC-PO KIT for mouse IgG (Vector Laboratories, Burlingame, CA) was employed for the avidin–biotin complex (ABC) method. Mouse monoclonal antibodies were diluted to 1:500 for PCNA (Dako Japan, Kyoto, Japan) or 1:1000 for cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated at 4 °C overnight. Then they were treated with biotin-labeled mouse IgG, ABC reagent, and visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemical Industries) with counter-staining by hematoxylin. To determine the PCNA and cyclin D1-positive indices in the lung, more than 1000 cells were counted in the randomly selected areas of normal alveolar epitheliums. To determine the PCNA positive index in the colon, at least 1000 epithelial cells were counted in the areas of well-visualized crypts from the proximal, middle and distal colonic portions. For all cases, indices were expressed as the percentages of positively-stained cells per totally-counted cells.

2.7. Immunohistochamistry for GST-P

In order to assess the rat liver pre-neoplastic lesions, sections ($n = 18–20$ in groups 1–3; $n = 5$ in groups 4 and 5) from three liver lobes (left, intermediate and caudate) were immunohistochemically stained for GST-P using anti-rabbit GST-P polyclonal antibodies (Medical & Biological Laboratories, Nagoya, Japan) at 1:1000 dilution, as described previously [17]. The numbers and the areas of GST-P foci 0.2 mm or more in diameter and the total areas of the HE-stained liver sections were measured with the aid of the Image Processor for Analytical Pathology (IPAP) system (Sumica Technos, Osaka, Japan).

2.8. Statistical analysis

Numerical values expressed as means ± SD were subjected to the *F*-test followed by the Student's or Welch's *t*-tests using StatLight software (Yukms Co, Tokyo, Japan). Differences in incidences of neoplastic and pre-neoplastic lesions were analyzed by the χ^2 or Fisher's exact provability test using Stat-View software (SAS Institute, Cary, NC). A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Animal condition, final body and organ weights, and intakes of water and diet

Although the modified DMBDD treatment conducted in this study was toxic, only 3 out of 60 animals became moribund or were found dead due to tumors. The remaining 57 DMBDD-treated rats were healthy until the end of the regimen at week 30, like the non-DMBDD-treated animals. Therefore, PJJ-34 did not affect survival of animals within this period.

Table 1 shows data for final body and organs weights and average intakes of water and diet. Final body weights in DMBDD groups 1–3 were not significantly different, and those in non-DMBDD groups 4 and 5 were also not significantly different. Relative liver, kidney and spleen weights also did not significantly vary among groups 1–3, or between groups 4 and 5. Therefore, PJJ-34 did not affect body and major organ weights. In addition, water intake and food consumption data did not differ among groups 1–3, or between groups 4 and 5. All these findings indicate that PJJ-34 did not show apparent toxicity and not affect survival of rats.

3.2. Chemopreventive effects of PJJ-34 on lung tumorigenesis

Macroscopically, all of the DMBDD-treated rats (groups 1–3) had many whitish nodular lesions on the surfaces of their lungs, most diagnosed as alveolar hyperplasias (100% incidence). The incidences (%) of neoplastic and pre-neoplastic lesions in the major targeted organs are summarized in Table 2. The majority of lung carcinomas were adenocarcinomas but a few included apparent squamous components (diagnosed as adeno-squamous carcinoma). The incidence of lung adenomas and carcinomas was significantly decreased by 5 mg/kg b.w. of PJJ-34 (group 2) as compared to the DMBDD-control (group 1) (adenoma, 100 → 75%; carcinoma, 63 → 30%; $P < 0.05$), but reduction in incidence was not apparent in the 10 mg/kg PJJ-34 case (group 3).

Fig. 2 shows the data for multiplicity (average number of lesions/rat) of neoplastic and/or pre-neoplastic lesions in the lung, colon and urinary bladder. Multiplicity of alveolar epithelial hyperplasias (Fig. 2a) was significantly decreased in both PJJ-34-treated rats relative to the non-treated controls ($P < 0.0001$). Multiplicity of adenomas was also significantly decreased ($P < 0.05$) in the 5 mg/kg (1.4 ± 1.1) and 10 mg/kg (1.7 ± 1.0) groups as compared to the DMBDD-alone group (2.5 ± 1.1). Multiplicity of carcinomas was significantly decreased only in group 2 (0.35 ± 0.59) as compared to group 1 (1.21 ± 1.65). Multiplicity of total tumors were significantly decreased in both groups 2 (1.8 ± 1.4, $P < 0.005$) and 3 (2.3 ± 1.4, $P < 0.05$) as compared to group 1 (3.7 ± 2.2). Thus, lung tumorigenesis was obviously suppressed by administration of PJJ-34 at both doses without a dose-dependency.

3.3. Chemopreventive effects of PJJ-34 on colorectal and bladder tumorigenesis

In the colorectum (Table 2), total tumor incidence was significantly decreased in group 3 (28%, $P < 0.05$) as compared to group 1 (63%). Although statistical significance was not detected, this was largely due to decrease in the adenocarcinoma incidence (47 → 17%). Similarly, as shown in Fig. 2b, multiplicities of adenocarcinomas (0.17 ± 0.38 vs. 0.47 ± 0.51) and total tumors (0.28 ± 0.46 vs. 0.68 ± 0.58) were signifi-

Table 1
Final body and relative organ weights, and daily intakes of water and diet.

Groups (treatment)	Effective o. of rats	Final body eights (g)	Relative organ weights (% of body weight)			Daily intakes (g/rat/day)[a]	
			Liver	Kidney	Spleen	Water	Diet
G1 (DMBDD → 0 mg/kg PJJ-34)	19	293 ± 20[b]	2.4 ± 0.4	0.63 ± 0.05	0.28 ± 0.09	16.7	11.6
G2 (DMBDD → 5 mg/kg PJJ-34)	20	295 ± 14	2.3 ± 0.2	0.63 ± 0.05	0.24 ± 0.04	15.9	11.5
G3 (DMBDD → 10 mg/kg PJJ-34)	18	296 ± 15	2.3 ± 0.3	0.65 ± 0.06	0.27 ± 0.12	16.7	11.8
G4 (Vehicle → 0 mg/kg PJJ-34)	5	361 ± 6	2.2 ± 0.1	0.54 ± 0.03	0.18 ± 0.01	18.7	13.2
G5 (Vehicle → 10 mg/kg PJJ-34)	5	348 ± 23	2.1 ± 0.1	0.55 ± 0.02	0.18 ± 0.01	18.3	12.8

PJJ-34 dissolved in 0.5% CMC-Na (carboxymethyl cellulose sodium salt) was intragastrically (i.g.) administered to rats 5 times per week in the post-initiation period. Body weights, water intake and food consumption were measured weekly until the 30-week termination.

[a] Data for average daily intakes of water and diet were reflected the measurements within PJJ-34-administered period.

[b] Means ± SD.

Table 2
Incidences of neoplastic and pre-neoplastic lesions in the target organs.

Organs	Pathological findings	G1: DMBDD → 0 mg/kg	G2: DMBDD → 5 mg/kg	G3: DMBDD → 10 mg/kg
Lung	Hyperplasia	100% (19/19)	100% (20/20)	100% (18/18)
	Adenoma	100% (19/19)	75% (15/20)*	94% (17/18)
	Carcinoma[a]	63% (12/19)	30% (6/20)*	50% (9/18)
	Total tumors	100% (19/19)	85% (17/20)	100% (18/18)
Duodenum	Adenocarcinoma	37% (7/19)	30% (6/20)	39% (7/18)
Jejunum	Adenocarcinoma	11% (2/19)	0% (0/20)	5.6% (1/18)
Colorectum	Adenoma	11% (2/19)	25% (5/20)	11% (2/18)
	Adenocarcinoma	47% (9/19)	30% (6/20)	17% (3/18)
	Mucinous carcinoma	0% (0/19)	5% (1/20)	0% (0/18)
	Signet-ring cell carcinoma	11% (2/19)	0% (0/20)	0% (0/18)
	Total tumors	63% (12/19)	50% (10/20)	28% (5/18)*
Liver	Hepatocellular adenoma	0% (0/19)	5% (1/20)	0% (0/18)
	Metastasis (mesenchymal tumor)	5.3% (1/19)	0% (0/20)	0% (0/18)
Kidney	Renal cell adenoma	0% (0/19)	10% (2/20)	5.6% (1/18)
	Nephroblastoma	42% (8/19)	35% (7/20)	56% (10/18)
Bladder	PN[b] hyperplasia	63% (12/19)	60% (12/20)	61% (11/18)
	Papilloma	16% (3/19)	10% (2/20)	5.6% (1/18)
	Transitional cell carcinoma	0% (0/19)	5% (1/20)	0% (0/18)
	Total tumors	16% (3/19)	15% (3/20)	5.6% (1/18)
Thyroid	Follicular cell adenoma	16% (3/19)	0% (0/20)	17% (3/18)
	Follicular cell carcinoma	11% (2/19)	5% (1/20)	5.6% (1/18)
	C-cell carcinoma	0% (0/19)	0% (0/20)	11% (2/18)
	Total tumors	26% (5/19)	5% (1/20)	33% (6/18)
Skin/subcutaneous tumors[c]		5.3% (1/19)	10% (2/20)	17% (3/18)

[a] Carcinomas in the lung include adenocarcinoma and adeno-squamous carcinoma.

[b] PN, papillary or nodular.

[c] Skin/subcutaneous tumors were each diagnosed as squamous cell papilloma, basal cell adenoma or carcinoma, and keratoacanthoma.

* $P < 0.05$ vs. corresponding group 1 (χ^2-test or Fisher's test).

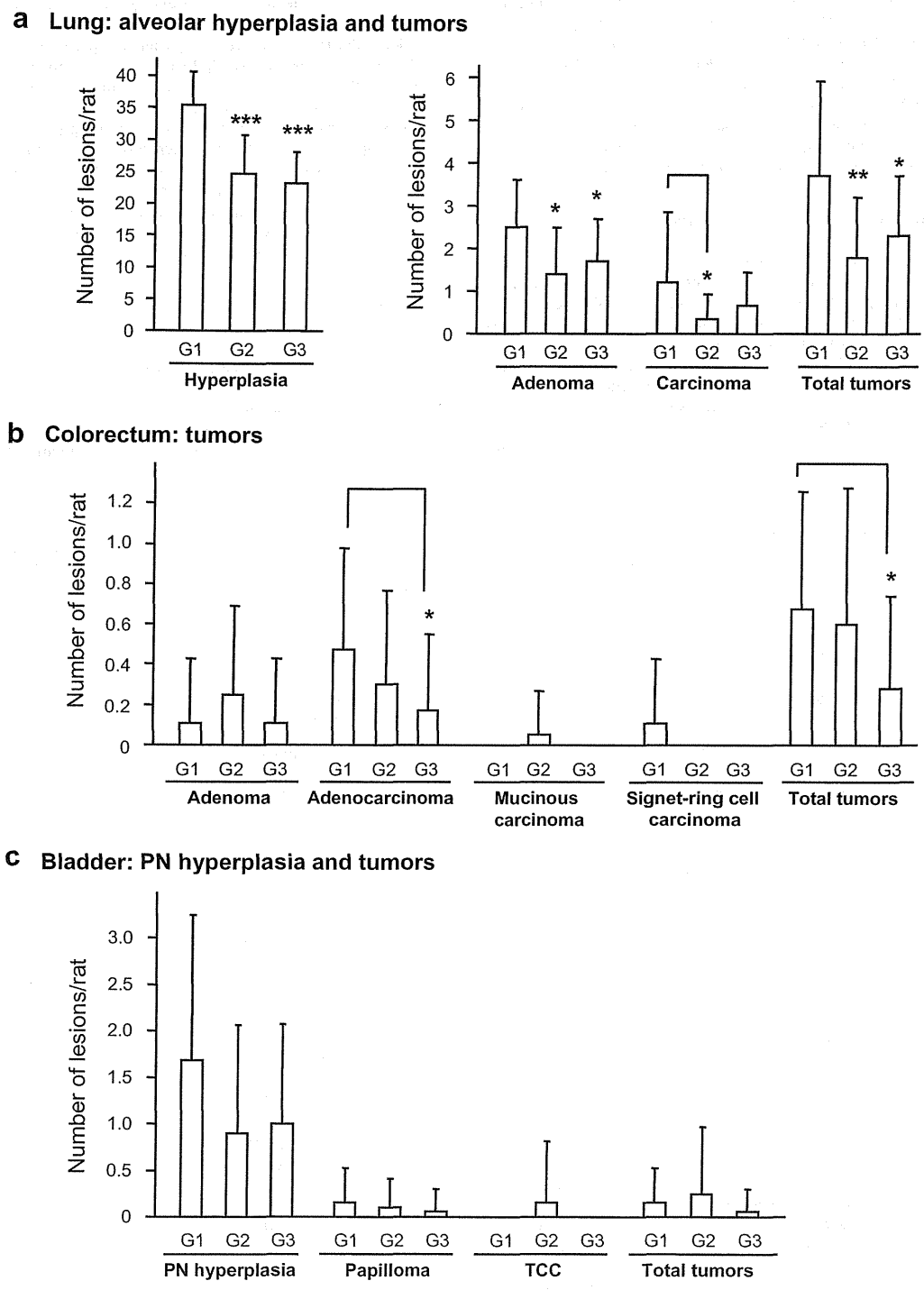

Fig. 2. Multiplicity (average number of lesions/rat) of neoplastic and pre-neoplastic lesions in the lung, colorectum and bladder (bars are SDs). (a) In the lung, multiplicities of alveolar hyperplasias, adenomas and total lung tumors were significantly decreased in groups 2 and 3, while that of carcinomas was only suppressed in group 2 as compared to group 1. (b) Total numbers of colorectal tumors were significantly decreased in group 3, as well as adenocarcinomas. (c) Among the bladder proliferative lesions, multiplicity of papillary or nodular (PN) hyperplasias tended to be decreased in groups 2 and 3 as compared to group 1. $^{*}P < 0.05$ vs. G1; $^{**}P < 0.005$ vs. G1; $^{***}P < 0.0001$ vs. G1.

cantly decreased in group 3 as compared to group 1 ($P < 0.05$) with a dose-dependency. On the other hand, incidences of bladder proliferative lesions (Table 2) were not significantly influenced by administration of PJJ-34. However, multiplicity of papillary or nodular (PN) hyperplasias tended to be decreased in both 5 and 10 mg/kg groups (Fig. 2c), without statistical significance.

3.4. Tumor incidences in the other organs

As shown in Table 2, the treatment of DMBDD can induce tumors at multiple-site at the whole-body level. Incidences of tumors in the major target organs (thyroid, kidney, liver, small intestine, skin/subcutis) other than the lung, colon and bladder were not apparently changed by administration of PJJ-34 as compared to the DMBDD-alone case.

3.5. Effect of PJJ-34 on cell proliferation in the lung and colon

Since tumor incidence and multiplicity of the lung and colon were significantly suppressed by PJJ-34, we further conducted immunohistochemical analysis of cell proliferation. As shown in Fig. 3a, PCNA-positive indices (%) in normal alveolar epitheliums of the lung were 6.9 ± 1.9, 3.7 ± 0.7 and 2.9 ± 0.4 in groups 1–3, respectively, and those for cyclin D1 were 3.8 ± 1.0, 2.7 ± 0.8 and 2.4 ± 1.0 in groups 1–3, respectively. Both indices were significantly suppressed by PJJ-34 (groups 2 and 3) as compared to group 1. On the other hand (Fig. 3b), PCNA-positive indices in normal colonic epitheliums were 33.9 ± 4.0, 30.7 ± 5.5 and 31.2 ± 4.0 in groups 1–3, respectively. Values were again significantly decreased in groups 2 and 3 as compared to group 1 but the differences were very small.

3.6. Quantitative analysis for GST-P foci in the liver

Fig. 4 shows quantitative data for GST-P foci (⩾0.2 mm in diameter) per liver area (/cm^2). The numbers were 8.4 ± 2.3 (group 1), 9.6 ± 4.4 (group 2) and 9.2 ± 2.9 (group 3), respectively, and the areas were 0.60 ± 0.22 (group 1), 0.70 ± 0.40 (group 2) and 0.65 ± 0.30 (group 3), respectively. There were no statistical significances among groups 1–3.

4. Discussion

The present study demonstrated a strong chemopreventive action of PJJ-34 against lung carcinogenesis and a relatively potent anti-rumor effect against colon carcinogenesis in rats. Suppression of cell proliferation appears to be an important chemopreventive action by PJJ-34. Regarding dose-efficacy, with an average human body weight of 60 kg, chemopreventive effect could be achieved at the relatively low levels of 300 mg/day/person (5 mg/kg b.w.) or 600 mg/day/person (10 mg/kg b.w.) and anti-carcinogenic effects of PJJ-34 noted in this study might not necessarily be dose-dependent. Interestingly, one natu-

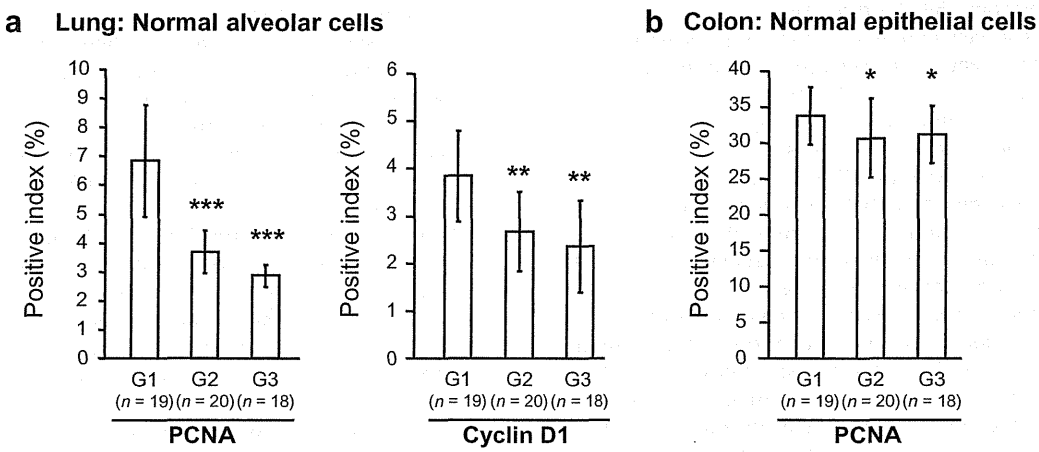

Fig. 3. Immunohistochemically demonstrated proliferation marker-positive indices (%) in the lung and colon (means ± SDs). (a) In normal alveolar epithelium of the lung, both PCNA- and cyclin D1-positive indices were significantly suppressed by PJJ-34 (5 and 10 mg/kg) as compared to the control. (b) In normal colonic epithelium, PCNA-positive indices were modestly but significantly suppressed by PJJ-34 (5 and 10 mg/kg). $^{*}P < 0.05$ vs. G1; $^{**}P < 0.0005$ vs. G1; $^{***}P < 0.0001$ vs. G1.

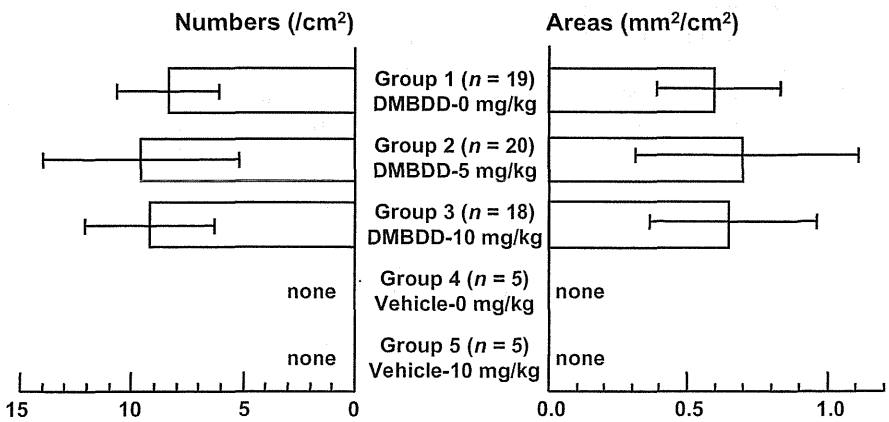

Fig. 4. Quantitative data for GST-P foci in the liver (means ± SDs). The numbers and the areas of GST-P foci 0.2 mm or more in diameter were quantitatively analyzed per liver area (/cm^2) and there was no significant variation in DMBDD-treated groups 1–3.

rally-occurring serratane-type triterpenoid, 3α-methoxyserrat-14-en-21β-ol (PJ-1), recently exhibited a similar inhibitory effect limited to lung carcinogenesis in the DMBDD model, without affecting other organs [20].

Until now, many naturally-occurring or synthetic serratane-type triterpenoids derived from the cuticle or stem bark of *P. jezoensis* Carr. var. *jezoensis* or *hondoensis* have proved to be potentially chemopreventive from EBV-EA testing assessed by Trypan-Blue staining (see Refs. by Tanaka et al.). In summary, at least 15 compounds out of above screened candidates have been evaluated for in vivo anti-tumor promoting effects in female ICR mice, including: PJJ-34 (5) and 3β-methoxy-21α-hydroxyserrat-14-en-29-al [5]; 14β,15β-epoxy-3β-methoxy-serratan-21β-ol [10]; 21-episerratenediol [11]; PJJ-1-13-en (4) and PJJ-43 (6) [12]; 13α,14α-epoxy-21α-methoxyserratan-3-one, 21α-methoxyserrat-13-en-3-one and 21α-hydroxy-3β-methoxyserrat-14-en-30-al [13]; PJJ-1 (1), 13α,14α-epoxyserratan-3β,21β-diol, 13α,14α-epoxyserratan-3α,21β-diol, 13α,14α-epoxy-3α,21β-dimethoxyserratane, 13α,14α-epoxy-3α,21β-diethoxyserratane and 14β-H-3α-methoxyserratan-15β,21β-diol [14]. All compounds demonstrated relatively potent anti-tumor effects in this established bioassay with a 20-week regimen in mouse. Among them, PJJ-34 most strongly reduced the incidence (100 → 20%) and multiplicity (9 → 0.8/mouse) of skin papillomas [5], which was therefore, subjected to the whole-body examination with the DMBDD bioassay.

It has been reported that anti-carcinogenic effects of triterpenoids are mainly due to anti-inflammatory, anti-proliferative and cytotoxic actions against tumor cells. For example, a steroid-like triterpenoid, oleanolic acid is a known chemopreventive agent used as a positive control in above-mentioned EBV-EA activation test and significantly reduced the numbers of azoxymethane (AOM)-induced aberrant crypt foci (ACF) in male F344 rats with significant reduction in the number of AgNORs in nuclei of the colonic epithelium [21]. Many kinds of pentacyclic triterpenes (PTs) have a wide distribution in plants and have been used as anti-inflammatory remedies in folk medicine. For example, oleanolic acid also possesses a strong anti-inflammatory potential [22]. In the present study, colorectal tumor numbers were significantly decreased in 10 mg/kg PJJ-34-administered rats mainly due to reduction of adenocarcinomas, suggesting that PJJ-34 may have efficiently suppressed the progression process from benign to malignant tumors in multi-step colon carcinogenesis. For one of the mechanisms, inhibition of cell proliferation (Fig. 3b) would be an essential role, as well as oleanolic acid [21]. However, another mechanism might also be operating. In fact, colon tumor incidence itself was obviously decreased in the 10 mg/kg group (63 → 28%) which may indicate that chemopreventive action of PJJ-34 is effective also in pre-neoplastic stages. In this context, PJJ-34 administered during the initiation period showed potent inhibition of mouse skin tumorigenesis with ultraviolet-B (UVB) initiation and TPA promotion [23]. Thus, the compound may possess both anti-initiation and anti-promotion/progression activities.

Nevertheless, the most prominent action of PJJ-34 resided in the marked inhibition of cell proliferation in normal alveolar cells of the lung (Fig. 3a). Regarding mechanisms, one synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), was found to inhibit cyclooxigenase-2 (COX-2) expression and demonstrated anti-proliferative activity with many human cancer cell lines [24]. Moreover, a recent study demonstrated CDDO to significantly inhibit lung adenocarcinoma development in female A/J mice treated with vinyl carbamate, and anti-inflammatory actions such as induction of heme oxygenase-1 (HO-1) and suppression of phosphorylation of signal transducers and activators of transcription 3 (STAT3), as well as induction of apoptosis, could be demonstrated in vitro [25]. Indeed, it is possible that anti-inflammatory, anti-oxidative and apoptotic effects may also participate in the mechanisms underlying anti-lung carcinogenesis. In addition, a further possible mechanism was suggested in an analogue of PJJ-34, namely PJ-1 [20]. PJ-1 demonstrated the lung-specific inhibition on DMBDD-induced multi-organ carcinogenesis with a significant reduction in PCNA-positive indices. The DMBDD treatment significantly decreased mRNA expression for cytochrome P450 (CYP) 2B1/2 in the rat lung and PJ-1 treatment significantly recovered their expression levels by which might read to activation of the detoxification process of a potent lung-targeting carcinogen, DHPN. Therefore, a similar mechanism might also relate to the PJJ-34 case. Anyway, further study is needed to clarify the underlying mechanisms of PJJ-34 against lung carcinogenesis.

From accumulated findings of chemical structural examination, some points should be noted regarding anti-carcinogenic effects of triterpenoids. First, biosynthetic alteration of the serratane skeleton indicates that the 6–6–7–6–6 ring system (serratane-type) effectively suppresses EBV-EA induction, while rearranged abeo-serratane skeletons, such as 6–6–7–5–6 [10,13] and 6–6–6–7–6 [8] ring systems have only reduced potency. For example, a naturally-occurring chemical analogue of PJJ-34, PJJ-43 (compound 6 in Fig. 1a) carries a different bonding of the epoxy ring at C-13 and C-14 (the epoxy epimer of PJJ-34), and this compound showed only weak anti-tumor effects (about 1/5 of that of PJJ-34) in a two-stage carcinogenesis test using mouse skin papillomas as endpoints. Therefore, the 13α,14α-epoxyserratane framework seems to be important for anti-tumor activity of these triterpenoids [12].

There are a lot of studies reporting significant cytotoxic effects of triterpenoids against a variety of tumor cell lines. For example, ursolic acid demonstrated significant cytotoxicity against lymphocytic leukemia cells P-388 and L-1210 as well as other human tumor cell lines [26], while also inhibiting lipoxygenase activity and HL-60 leukemic cell proliferation [27]. Besides, DMBA-initiated, TPA-promoted mouse skin tumorigenesis was also significantly suppressed by treatment with ursolic acid in female CD-1 mice [28]. Other triterpenoids such as hederagenin and its 3-*O*-glycosides (kalopanaxsaponin A and I) were also found to be cytotoxic to various tumor cell lines, including P-388, L-1210, HL-60, U-937, HepG-2 and SNU-C5, and anti-mutagenic against aflatoxin B1 (AFB1), possibly through inhibition of mutagenic activation of the carcino-

gen [29]. In addition, a report has appeared indicating that the coumaroyl moiety at the C-3 position of lupine-type triterpenes (such as 3-*O*-p-coumaroylalphitolic acids) may play a key role in enhancing cytotoxic activity against tumor cell lines [30].

In conclusion, a novel serratane-type triterpenoid, PJJ-34, is a chemopreventive agent against lung and colon carcinogenesis in the post-initiation phase in DMBDD-treated rats, without any apparent toxicity at the body or organ levels. Suppression of cell proliferation could be an important mode of action of this compound and further studies to elucidate underlying mechanisms appear warranted.

Conflict of interest

The authors disclose no potential conflicts of interest to the present work.

Acknowledgments

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