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Reduced serum vascular endothelial growth factor receptor-2 (sVEGFR-2) and sVEGFR-1 levels in gastric cancer patients

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The relationship between gastric cancer and serum vascular endothelial growth factor receptor-1 (sVEGFR-1) and sVEGFR-2, which are soluble form receptor proteins of vascular endothelial growth factor (VEGF), has not been extensively studied. VEGF, sVEGFR-1 and sVEGFR-2 were measured in the sera obtained before surgical operation from 164 gastric cancer patients and from 164 healthy controls matched for age and gender. Compared with controls, the cases showed elevated VEGF ($P < 0.01$) and reduced sVEGFR-1 ($P = 0.07$) and sVEGFR-2 ($P = 0.02$). The difference in VEGF levels was small among men and when the outcome was early cancer. The difference in sVEGFR-1 levels was significant or borderline significant only in men and when the outcome was diffuse type cancer. The difference in sVEGFR-2 levels was significant only in men and when the outcome was advanced or diffuse type cancer. The sensitivities and specificities of VEGF, sVEGFR-1 and sVEGFR-2 were all approximately 60%. For diffuse type cancer, sVEGFR-2 showed a sensitivity of 62.4% and a specificity of 63.4%, which was similar to serum pepsinogen. In conclusion, elevated VEGF and reduced sVEGFR-1 and sVEGFR-2 in serum are characteristic of gastric cancer patients, and the value of serum sVEGFR-2 in the diagnosis of diffuse type gastric cancer should be further evaluated. (*Cancer Sci* 2011; 102: 866–869)

Although the incidence and mortality of gastric cancer have been declining among the younger generation in Japan,[1] it remains as the second highest cause of cancer death.[2] Recently, it has been proposed that serum *Helicobacter pylori* antibody and pepsinogen values should be used for risk assessment of gastric cancer in adults,[3,4] and gastric cancer prevention programs are expected to become more cost-effective, if the programs do not target at subjects without *H. pylori* infection or abnormal serum pepsinogen values, who have very low risks of gastric cancer.[4,5] However, the sensitivity of serum pepsinogen is poor for diffuse type gastric cancer.[6] Vascular endothelial growth factor (VEGF) is a factor promoting vascularization, and it plays a role in both physical and malignant conditions.[7] Staining with VEGF antibodies has revealed the presence of VEGF in some malignant tissues.[8] Previous studies have shown that serum VEGF concentration is high in several cancers, such as breast and colon cancers.[9,10] In gastric cancer patients, elevated VEGF predicts a poor prognosis[8,11] and is often accompanied by other malignant factors such as TGFβ-1.[12]

The biological effects of VEGF are mediated by two receptor tyrosine kinases, VEGFR-1 and VEGFR-2, which are almost exclusively expressed within endothelial cells. In addition to VEGFR-1 and VEGFR-2, a soluble form of VEGF-R1 (sVEGFR-1), a naturally occurring and alternatively spliced variant, functions as a high-affinity receptor of VEGF. Compared to VEGFR-1, VEGFR-2 is more widely distributed and expressed in all vessel-derived endothelial cells. The VEGF/VEGFR-2 signaling pathway plays a crucial role in tumor angiogenesis. Although the exact role of VEGFR-1 remains controversial, the available evidence have shown that VEGFR-1 functions to limit VEGF/VEGFR-2 mediated angiogenesis with intact receptor acting as a decoy and soluble form creating inert receptors by dimerization with VEGFR-2 or sequestering free ligand.[7] Soluble form of VEGFR-2 (sVEGFR-2) as well as sVEGFR-1 can be detected in serum. Circulating VEGF is known to be higher in gastric cancer patients than in healthy subjects.[13] However, the data on circulating sVEGFR-1 and sVEGFR-2 levels are limited.

In this study, serum VEGF, sVEGFR-1 and sVEGFR-2 levels were compared between gastric cancer patients and matched healthy controls. Secondary analyses examined different subtypes of gastric cancer defined by progression stage and histopathological type. To evaluate the diagnostic accuracy of the VEGF, sVEGFR-1 and sVEGFR-2 levels, the optimal cutoff values, sensitivities and specificities for gastric cancer were calculated.

Materials and Methods

Subjects in this study were originally enrolled in our previous study; the details of the subject recruitment and data collection are provided elsewhere.[14,15] Briefly, sera were collected from 787 gastric cancer patients who were younger than 70 years of age and were admitted to the surgical division of nine hospitals in the Tokyo Metropolitan Area between June 1993 and July 1995. Phlebotomy of each patient was performed before cancer treatment (surgical operation or chemotherapy). The sera were also collected from 1007 apparently healthy subjects who were admitted for health screening programs between June 1993 and November 1994. Informed consent was obtained from all subjects. The diagnosis of cancer was confirmed, and other information, including histological types and progression stages, was collected from histopathological reports for resected or biopsy specimens.

In three of the nine hospitals, prognosis information for gastric cancer patients was available. Of the 571 patients from these three hospitals, 198 cases were randomly selected so that young patients were included, and the proportion of men and women were similar in each 10-year age group.

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Table 1. Characteristics of subjects

	Control	Case	P-value
Number of subjects	164	164	
Age (years)	53.9 ± 10.0*	54.0 ± 9.9*	Matched factor
Male/Female	78/86	78/86	Matched factor
Smoking dose (number of cigarettes per day multiplied by smoking years)			
No smoking history	84 (51.2%)	81 (49.4%)	$P = 0.43$
1–399	27 (16.5%)	23 (14.0%)	
400–799	22 (13.4%)	25 (15.2%)	
800+	21 (12.8%)	30 (18.3%)	
Unknown	10 (6.1%)	5 (3.0%)	
Drinking dose (amount of alcohol consumed [g] per week multiplied by drinking years)			
No drinking history	47 (28.7%)	54 (32.9%)	$P < 0.01$
Occasional/1–134.9	38 (23.2%)	28 (17.1%)	
135–1349.9	35 (21.3%)	27 (16.5%)	
1350+	19 (11.6%)	41 (25.0%)	
Unknown	25 (15.2%)	14 (8.5%)	
Helicobacter pylori seropositive†	105 (64.0%)	159 (97.0%)	$P < 0.01$

*Mean ± standard deviation. †Measured using J-HM-Cap (Kyowa Medex Co. Ltd., Tokyo).

A healthy control was matched to each case based on age (within 2 years) and gender. In 34 pairs, the serum sample from either the case or the control had already been used up, so data on 164 matched pairs were available for this study. Based on the pathological information, cases were classified into early (depth of invasion was within submucosa) and advanced (depth of invasion includes propria muscle) gastric cancer or into intestinal and diffuse type cancers.

VEGF, VEGFR-1 and VEGFR-2 were measured in the sera with the commercial ELISA kit Quantikine from R&D systems (Minneapolis, MN, USA) for human VEGF, sVEGF R1 and sVEGF R2 by a researcher who was blind to the case status associated with the samples. Serum VEGF, sVEGFR-1 and sVEGFR-2 levels were compared between cases and controls by paired *t*-tests. To evaluate the diagnostic accuracy of the factors, the optimal cutoff values and the sensitivity and specificity were calculated for all, early, advanced, intestinal and diffuse type cancers. In these calculations, the controls were restricted to those who were paired to cases belonging to the specific classification.

Results

Table 1 shows the characteristics of the subjects. Although no remarkable difference in smoking was observed between cases and controls, the cases drank more alcohol and had a higher prevalence of *H. pylori*. VEGF and sVEGFR-2 levels were measured for all subjects, but the sVEGFR-1 level was measured only in 147 pairs because of insufficient sera.

The VEGF level was higher in cases than in controls (Tables 2,3), but the difference was weak among men and when the outcome was early cancer. Compared with controls, the cases tended to have lower sVEGFR-1 levels, and the difference was significant among male subjects and borderline significant when the outcome was diffuse type cancer. The sVEGFR-2 level was lower in cases of advanced or diffuse-type cancer than in their matched controls.

Table 4 shows the optimal cutoff values for VEGF, sVEGFR-1 and sVEGFR-2. Of the three factors, VEGF showed the best sensitivity and specificity, 63.5% and 65.1%, respectively, for intestinal-type cancer when the cut-off value was 415 pg/mL. sVEGFR-2 gave the best diagnostic accuracies for all advanced and diffuse type cancers where cut-off values (sensitivities and specificities) were 8520 pg/mL (61.0% and 60.4%), 8314 pg/mL (66.3% and 61.6%) and 8520 pg/mL (62.4% and 63.4%), respectively. For early gastric cancer, sVEGFR-1 gave the best sensitivity and specificity, 60.0% and 58.6%, respectively, when the cut-off value was 46.0 pg/mL.

Table 2. VEGF, sVEGFR-1 and sVEGFR-2 levels in cases and controls

	VEGF (pg/mL) Mean ± SD	sVEGFR-1 (pg/mL) Mean ± SD	sVEGFR-2 (pg/mL) Mean ± SD
Total			
Number of pairs	164	147	164
Controls	479.1 ± 350.8	56.96 ± 34.34	8853 ± 1888
Cases	640.6 ± 516.8	48.47 ± 32.45	8397 ± 2014
P-value*	$P < 0.01$	$P = 0.07$	$P = 0.02$
Male			
Number of pairs	78	71	78
Controls	511.6 ± 372.4	56.10 ± 36.26	9304 ± 1822
Cases	649.3 ± 517.6	42.58 ± 30.46	8343 ± 2186
P-value*	$P = 0.06$	$P = 0.02$	$P = 0.04$
Female			
Number of pairs	86	76	86
Controls	479.6 ± 329.4	55.82 ± 32.69	8443 ± 1864
Cases	632.8 ± 519.0	53.97 ± 33.47	8173 ± 1828
P-value*	$P < 0.01$	$P = 0.74$	$P = 0.28$

*Results from paired *t*-tests. VEGF, vascular endothelial growth factor; sVEGFR-1/2, serum vascular endothelial growth factor receptor-1/2.

Table 3. VEGF, sVEGFR-1 and sVEGFR-2 levels in cases and controls with respect to progression stage (early or advanced) and histopathological type (intestinal or diffuse)

	VEGF (pg/mL) Mean ± SD	sVEGFR-1 (pg/mL) Mean ± SD	sVEGFR-2 (pg/mL) Mean ± SD
Early gastric cancer (depth of tumor invasion is within submucosa)			
Number of pairs	78	70	78
Controls	485.4 ± 344.0	52.68 ± 30.79	8853 ± 1770
Cases	607.4 ± 422.8	46.43 ± 28.81	8811 ± 2075
P-value*	$P = 0.06$	$P = 0.24$	$P = 0.88$
Advanced gastric cancer (depth of invasion includes propria muscle)			
Number of pairs	86	77	86
Controls	473.3 ± 358.7	58.93 ± 37.23	8852 ± 2001
Cases	670.8 ± 590.2	50.32 ± 35.53	8021 ± 1891
P-value*	$P < 0.01$	$P = 0.16$	$P < 0.01$
Intestinal type gastric cancer			
Number of pairs	63	57	63
Controls	474.5 ± 391.3	50.64 ± 32.38	8501 ± 1764
Cases	658.6 ± 541.3	47.28 ± 30.96	8556 ± 2208
P-value*	$P = 0.03$	$P = 0.58$	$P = 0.87$
Diffuse type gastric cancer			
Number of pairs	101	90	101
Controls	481.9 ± 325.0	59.32 ± 35.29	9072 ± 1938
Cases	629.4 ± 503.4	49.22 ± 33.51	8297 ± 1887
P-value*	$P = 0.02$	$P = 0.06$	$P < 0.01$

*Results of paired *t*-tests. VEGF, vascular endothelial growth factor; sVEGFR-1/2, serum vascular endothelial growth factor receptor-1/2.

Discussion

VEGF was elevated in gastric cancer patients compared to controls. The presence of VEGF in a gastric cancer lesion[11] and a

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Table 4. Optimal cutoff values (sensitivity, specificity) of the factors for all, early, advanced, intestinal type and diffuse type cancers

Factor	All	Early	Advanced	Intestinal	Diffuse
VEGF (pg/mL)*	420 (60.4%, 57.9%)	428 (61.5%, 56.4%)	420 (58.1%, 60.5%)	415 (63.5%, 65.1%)†	428 (56.4%, 54.5%)
sVEGFR-1 (pg/mL)‡	45.6 (57.8%, 57.1%)	46.0 (60.0%, 58.6%)†	45.5 (57.1%, 55.8%)	40.7 (50.9%, 57.9%)	45.8 (58.9%, 60.0%)
sVEGFR-2 (pg/mL)‡	8520 (61.0%, 60.4%)†	8959 (55.1%, 52.6%)	8314 (66.3%, 61.6%)†	8400 (58.7%, 57.1%)	8520 (62.4%, 63.4%)†

*Positive is defined as the marker level being greater than or equal to the value given. †The best sensitivity and specificity of the three markers. ‡Positive is defined as the marker level being less than the value given. VEGF, vascular endothelial growth factor; sVEGFR-1/2, serum vascular endothelial growth factor receptor-1/2.

high level of circulating VEGF indicate a poor prognosis.[16–19] The result of the current study on VEGF is consistent with the results of previous studies.[13,20,21] The VEGF may have originated from gastric cancer cells[8] and the production and secretion may be increased with the progression of the cancer, but whether the cancer is intestinal or diffuse may have little influence on the VEGF level. It has been reported that *H. pylori* infection elevates serum VEGF level,[22,23] which can be a reason for the elevated VEGF level in gastric cancer patients. However, no association was observed between *H. pylori* serology and VEGF, sVEGFR-1 or sVEGFR-2 level in controls of this study.

Compared to the controls, the sVEGFR-1 and sVEGFR-2 levels were reduced in gastric cancer patients. One possibility is that the antibody used for ELISA recognizes the same or a near region as the ligand binds. Elevated VEGF levels may bind to these receptors and thereby reduce the sVEGFR-1 and sVEGFR-2 levels. Vascularization may promote cancer progression, and soluble VEGFR-1 and VEGFR-2 may act as decoys and disturb the binding of VEGF to VEGFR-2 on the surface of target cells. Reduced VEGFR-1 and VEGFR-2 levels and an elevated VEGF level stimulate the progression of gastric cancer and thus may be characteristic in gastric cancer patients.

Compared with VEGF, limited studies have examined circulating sVEGFR-1 level for gastric cancer and their role remains elusive. Colorectal cancer patients showed lower serum sVEGFR-1 level than controls did,[24] which is consistent with this study. Studies on pancreatic and biliary tract cancers gave similar results with this study on serum VEGF levels, but they showed higher sVEGFR-1 levels in patients than in controls.[25,26] Several studies to date have investigated relationships between sVEGFR-1 levels and prognosis as for several sites of cancers, which is inconsistent.[27] On sVEGFR-2 level, studies have been more limited. Further studies are warranted to clarify the role of sVEGFR-1 and sVEGFR-2 and their interactions with VEGF in the development of gastric cancer. The difference in the levels of the three factors between gastric cancer patients and controls was affected by gender, progression stage and histopathological type. The difference in the sVEGFR-1 level was not as clear as that for sVEFGR-2, which may be due to the obscure role of VEGFR-1 in vascularization. The differences in the VEGF and sVEGFR-2 levels were more striking for advanced cancer than early cancer, which can be explained by greater VEGF secretion from advanced gastric cancer tissues and by the binding of circulating sVEGFR-2. The difference in VEGF levels between matched cases and controls was smaller among men than women, while the differences in sVEGFR-1 and sVEGFR-2 levels between matched cases and controls were greater in men than in women. The underlying reason for the gender difference is unknown, but hormonal differences could exert some effect.

The difference in VEGF between matched cases and controls was similar between the intestinal and diffuse type cancers, whereas the difference in sVEGFR-1 and sVEGFR-2 levels was greater in the diffuse type cancer than in intestinal cancer. An explanation for the reduced sVEGFR-2 level in diffuse type gastric cancer is that advanced cancer was more frequent in diffuse type cancer than in the intestinal type. Actually, 54% of early and 69% of advanced cancers were diffuse types ($P = 0.06$). TGF-β1 is upregulated in patients with diffuse type gastric cancer[28,29] and TGF-β1 downregulates the expression of VEGFR-2 in endothelial cells.[30] These facts may be associated with the reduced sVEGFR-2 level in diffuse type gastric cancer. Serum pepsinogen II showed a sensitivity of 83.3% and a specificity of 76.9% for gastric cancer among those younger than 40 years of age,[31] although the sensitivity and specificity were weaker in those over 40 years. The sensitivities and specificities for the optimal cut-off values of VEGF, sVEGFR-1 and sVEGFR-2 were all approximately 60%, which is a somewhat unsatisfactory level. However, there were two interesting findings. One was that sVEGFR-2 showed a relatively good diagnostic accuracy compared with VEGF, although with VEGF, there was a smaller P-value than sVEGFR-2 in the paired t-test between cases and controls. The other finding was that sVEGFR-2 gave a similar diagnostic accuracy for diffuse type gastric cancer than serum pepsinogen. Serum pepsinogen, which is a good marker for gastric cancer and its risk,[32] does not show a good diagnostic accuracy for the diffuse type cancer.[6] When the diagnostic accuracy of serum pepsinogen for diffuse type cancer was calculated, and a positive result was defined as a pepsinogen I concentration not more than 70 ng/mL and the pepsinogen I to II ratio not more than 3.0,[33] the sensitivity and specificity were 65.3% and 59.4%, respectively. Compared with these values, the values for sVEGFR-2 of 62.4% and 63.4% were similar. Serum sVEGFR-2 can be used in the diagnosis of gastric cancer as the diagnostic accuracy of serum pepsinogen is not excellent. However, cautions are needed when using it to detect gastric cancer, because serum sVEGFR-2 is not specific for gastric cancer. The value of serum sVEGFR-2 in the diagnosis of gastric cancer, especially of the diffuse type, should be evaluated in further studies.

Because this study was not prospective, the differences in the serum levels of the markers may have been due to gastric cancer, and thus, the evaluation of the markers as risk indicators was impossible. We took this into consideration when interpreting the results. Another weakness of our study was that the measurement of the marker levels in the sera was performed after several years of frozen preservation. The preservation condition was not different between the cases and controls, and the measurement was performed by a researcher who was blinded to the case status for each serum sample. Thus, neither bias nor a difference in the preservation condition was expected to distort the results.

In conclusion, serum VEGF was elevated and sVEGFR-1 and sVEGFR-2 levels were reduced in gastric cancer patients. The difference in the levels of the three factors between gastric cancer patients and controls was affected by gender, progression stage and histopathological type. sVEGFR-2 showed a sensitivity and specificity for predicting diffuse type cancer that was similar to that of serum pepsinogen.

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Disclosure Statement

The authors have nothing to declare as financial disclosures.

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Effect of coffee consumption on all-cause and total cancer mortality: findings from the JACC study

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Abstract Coffee consumption is known to be related to various health conditions. Recently, its antioxidant effects have been suggested to be associated with all-cause or cancer mortality by various cohort studies. However, there has been only one small Asian cohort study that has assessed this association. Thus, we tried to assess the association of coffee with all-cause and total cancer mortality by conducting a large-scale cohort study in Japan. A total of 97,753 Japanese men and women aged 40–79 years were followed for 16 years. Hazard ratios and 95% confidence intervals of all-cause and total cancer mortality in relation to coffee consumption were calculated from proportional-hazards regression models. A total of 19,532 deaths occurred during the follow-up period; 34.8% of these deaths were caused by cancer. The all-cause mortality risk decreased with increasing coffee consumption in both men and women, with a risk elevation at the highest coffee consumption level (≥4 cups/day) compared with the 2nd highest consumption level in women, although the number of subjects evaluated at this level was small. No association was found between coffee consumption and total cancer mortality among men, whereas a weak inverse association was found among women. The present cohort study among the Japanese population suggested that there are beneficial effects of coffee on all-cause mortality among both men and women. Furthermore, the results showed that coffee consumption might not be associated with an increased risk of total cancer mortality.

Keywords All-cause mortality · Coffee consumption · Cohort study · Total cancer mortality

Abbreviations

95% CI	95 percent confidence interval
BMI	Body mass index
HR	Hazard ratio
JACC Study	Japan Collaborative Cohort Study for Evaluation of Cancer Risk
MI	Myocardial infarction

The member list of the JACC study group are listed in the "Appendix".

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Introduction

Coffee contains a variety of biological compounds such as caffeine, caffeic acid, chlorogenic acid and diterpenes. Caffeine is known to stimulate tumors, inhibit insulin activity, increase blood pressure and increase homocysteine level, and all of these effects may be harmful to health. However, coffee also has beneficial effects in that it can prevent cancer and inflammation thorough its antioxidant activity. Moreover, it is suggested that caffeic acid inhibits DNA methylation, chlorogenic acid improves glucose

tolerance, diterpenes have anticarcinogenic properties, and various components of coffee may be associated with favorable effects on health [1, 2].

Studies examining the risk of coffee consumption and its association with all-cause mortality have produced inconsistent results. Some cohort studies have found an inverse association [3–5] with all-cause mortality, while other studies have found positive [6, 7] and U-shaped associations [8, 9]. Furthermore, data on coffee consumption in relation to total cancer mortality is sparse [3, 10, 11]. Several epidemiologic studies have examined coffee consumption and the risk of cancer by site, such as the liver [12, 13], kidney [14] and breast [15]. These studies mainly showed an inverse association with increasing consumption of coffee; however, the results of studies on other cancer sites have been inconsistent. There are several cohort studies from Asian countries that have confirmed the associations between coffee consumption and the risk of site-specific cancer [12, 13, 16, 17]; however, to the best of our knowledge, only one cohort analysis (with 2,855 subjects) has investigated the impact of coffee consumption as a risk factor for all-cause and total cancer mortality [3]. The objective of this study was to assess the association of coffee with all-cause and total cancer mortality using a large-scale cohort study in Japan with a follow-up period of 16 years.

Methods

Study subjects and data collection

The Japan Collaborative Cohort Study for Evaluation of Cancer Risk (JACC study) was started between 1988 and 1990, enrolling subjects living in 45 areas in Japan. Sampling methods and details of the JACC study have been described elsewhere [18]. Subjects were recruited mainly at the time of their health-checkup using a self-administered questionnaire and a response rate of 83% was obtained. We followed 110,792 subjects (46,465 men and 64,327 women), aged 40–79 years at baseline.

Information about coffee consumption and other lifestyles was obtained using the self-administered questionnaire. Subjects were grouped into four categories according to their daily coffee intake at baseline: those consuming less than 1 cup, 1 cup, 2–3 cups, or 4 or more cups. The question regarding coffee consumption was assessed previously by a validation study and a high agreement with 12-day weighted dietary records was reported (Spearman correlation: 0.81) [19]. A total of 97,753 individuals (40,672 men and 57,081 women) provided responses to the coffee consumption question, and were included in the analysis.

Follow-up

The date and cause of deaths were confirmed, with the permission of the Director-General of the Prime Minister's Office, up to the end of 2006 by death certificates, except in seven areas where follow-ups were discontinued at the end of 1999 or 2003. Individuals who moved away from the study area were treated as study dropouts because deaths after such moves could not be confirmed in our follow-up system. Our entire study design was approved by the Ethical Board of Nagoya University School of Medicine, where the central secretariat of the JACC study was located.

Analysis

The distribution of some socio-demographic factors was compared between different coffee consumption groups using ANOVA or the Mantel–Haenszel test adjusted for age category. Hazard ratios (HRs) were calculated by Cox's proportional hazard model adjusted for 5-year age groups separately by gender. In multivariate analyses, we further adjusted several factors which were known to be associated with all-cause mortality and/or coffee consumption–that is, smoking status (current smoker, former smoker, or never smoker), drinking status (current drinker, former drinker, or never drinker), daily walking duration (walking more than 1 h per day or not), sleep length (< 6.5 h, 6.5–8.4 h, ≥8.5 h), consumption of green-leafy vegetables (almost daily or not), green tea consumption (daily or not), perceived stress (yes or no), education (attended school up to 15–18, >18 years old), body mass index (BMI; < 18.5, 18.5–24.9, ≥ 25.0), marital status (having a spouse or not) or disease history (cancer, myocardial infarction, stroke, or none). Data for the above factors was self-reported. For all covariates, missing values were treated as an additional category in the model. The linear trend in mortality risk was assessed by treating the number of cups of coffee intake per day as an ordinal variable. Further analysis stratified by baseline age (40–59 years and 60–79 years) in addition to gender was also conducted. To evaluate reverse causation, the risk of mortality, excluding subjects with past history of cancer, MI or stroke, or deaths occurring every 2 years up to 8 years (half of median follow-up period) from baseline, was also estimated. All statistical analyses were performed using the Statistical Analysis System (SAS 9.1, Cary, NC) at the Aichi Medical University Computer Center.

Results

During the 16-year median follow-up period, 4,876 subjects (1,766 men and 3,110 women) dropped out of the

follow-up while 19,532 deaths (11,178 men and 8,354 women) occurred. Of these deaths, 34.8% (37.3%, men; 31.5%, women) were caused by cancer, followed by cardiovascular diseases (30.7%, total; 28.3%, men; 34.0%, women). Of men's cancer mortality, the five most common sites were the lung, stomach, liver, pancreas and colon (23.4, 19.3, 11.3, 5.7 and 5.7% of men's cancer deaths, respectively). Of women's cancer mortality, the five most common sites were the stomach, lung, pancreas, liver and colon (15.9, 11.9, 9.5, 9.3 and 9.2%, respectively).

As shown in Table 1, those who consumed high amounts of coffee were younger, more likely to be a smoker, educated, highly stressed, and less likely to drink green tea and have a past disease history; this applied to both men and women.

The HRs of all-cause and total cancer mortality based on coffee consumption are shown in Table 2. The risk of all-cause mortality decreased with increasing coffee consumption in both men and women; however, in women, there was a slight risk elevation at the highest coffee consumption level compared with the 2nd level. Multivariable-adjusted HRs of those who consumed 4 or more cups of coffee per day were 0.80 (95% CI: 0.68–0.95) in men and 0.89 (0.66–1.20) in women compared with those who consumed less than 1 cup of coffee per day. The risk of total cancer mortality did not show any association with coffee consumption in men, but was slightly reduced with increasing coffee consumption in women, with a significant decreasing trend. Table 3 shows the results of analysis performed separately for each age group. Although coffee drinkers were more apparent in the younger age group than in the older age group, the decreasing trend of all-cause mortality with increasing coffee consumption was apparent in either age group. For total cancer mortality risk, men of both age groups showed no association with coffee consumption. Women aged 40–59 at baseline showed a slightly non-significant decreasing trend with increased coffee consumption, whereas women aged 60–79 at baseline showed risk reduction in only the 2nd and 3rd levels.

The results of further analysis, with exclusion of those with past medical history or those who died early, to examine reverse causation for coffee consumption and mortality are shown in Table 4. Analysis performed excluding those with past history of cancer, MI or stroke showed no remarkable alteration of the results among both men and women. Sequent exclusion of deaths that occurred within 2–8 years from baseline did not change the association of coffee consumption with all-cause and total cancer mortality in men. In women, the all-cause mortality risk reductions of consumers of 1 cup or 2–3 cups of coffee per day were weakened and consumers with the highest consumption level showed HRs greater than 1.00 after the

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Table 1 Baseline characteristics by coffee consumption

		Men					Women				
		<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	P	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	P
Participants	N	29,077	4,502	6,002	1,091		41,095	8,037	7,183	766	
Age (mean)	year	58.9	57.2	53.9	51.1	<0.0001	59.4	56.9	53.3	49.7	<0.0001
Current smoker	%	47.8	53.4	66.9	81.0	<0.0001	3.9	5.6	10.0	30.0	<0.0001
Current drinker	%	75.9	76.0	73.1	63.0	<0.0001	20.9	28.8	33.9	34.5	<0.0001
Walking 1 h/day ≤	%	50.8	47.6	46.4	44.7	<0.0001	51.5	50.4	51.2	50.6	0.27
Sleep 6.5–8.4 h/day	%	70.0	73.5	73.1	67.8	0.93	67.0	67.3	64.1	50.8	<0.0001
Eat green-leafy vegetables almost daily	%	26.2	29.0	26.4	23.0	<0.0001	30.6	34.4	32.8	32.3	<0.0001
Drink green tea daily	%	84.4	85.5	81.1	75.5	<0.0001	83.0	81.1	77.0	67.6	<0.0001
BMI (age-adjusted mean)	kg/m^2	22.5	22.5	22.4	22.2	<0.0001	22.8	22.7	22.7	22.3	<0.0001
Attend school up to 18 years old	%	12.2	19.8	21.2	25.0	<0.0001	7.1	10.9	12.5	14.6	<0.0001
Mentally stressed	%	19.7	24.4	30.5	38.3	<0.0001	18.4	20.4	25.9	29.9	<0.0001
Having a spouse	%	93.4	94.5	93.8	92.0	0.86	81.2	83.9	86.5	83.8	<0.01
Past history of cancer, MI or stroke	%	7.1	6.5	5.0	2.4	0.54	6.6	7.0	4.8	4.3	<0.0001

P values were calculated by ANOVA or Mantel–Haenszel test adjusted for 5-year age groups

BMI body mass index, *MI* myocardial infarction

Table 2 Hazard ratios for all-cause and total cancer mortality by coffee consumption

	Men					Women				
	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	Trend *P*	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	Trend *P*
Person-years	413,510	61,914	83,801	15,545		613,607	111,465	100,872	10,944	
All causes										
Cases	8,989	1,035	1,004	150		7,098	738	474	44	
Age-adjusted HR (95% CI)	1.00	0.94 (0.88–1.00)	0.90 (0.84–0.96)	0.89 (0.76–1.05)	<.01	1.00	0.81 (0.75–0.87)	0.83 (0.76–0.91)	1.03 (0.7–1.38)	<.0001
Multivariable-adjusted HR (95% CI)	1.00	0.95 (0.89–1.01)	0.86 (0.81–0.93)	0.80 (0.68–0.95)	<.0001	1.00	0.82 (0.76–0.89)	0.83 (0.75–0.91)	0.89 (0.66–1.20)	<.0001
Total cancer										
Cases	3,246	408	436	76		2,142	278	191	17	
Age-adjusted HR (95% CI)	1.00	0.99 (0.90–1.10)	1.00 (0.90–1.11)	1.15 (0.91–1.44)	0.27	1.00	0.90 (0.79–1.02)	0.87 (0.75–1.02)	0.94 (0.58–1.51)	0.05
Multivariable-adjusted HR (95% CI)	1.00	1.02 (0.92–1.13)	0.97 (0.88–1.08)	1.06 (0.84–1.33)	0.74	1.00	0.89 (078–1.01)	0.85 (0.73–0.99)	0.81 (0.50–1.32)	0.01

Multivariable-adjusted HR; adjusted for age categories, smoking status, alcohol drinking, walking hours, sleep duration, body mass index, consumption of green-leafy vegetables, green tea consumption, education, stress, marital status, past history of cancer, myocardial infarction or stroke

exclusion of deaths, which occurred within 6–8 years from baseline. The inverse association of total cancer mortality in women was weakened and was no longer statistically significant after the exclusion of deaths that occurred within 6 years.

Discussion

The results suggest the beneficial effects of coffee on all-cause mortality in both men and women. For women, although the number of subjects in the highest level was small, the change of the RR by increasing level coffee consumption may suggest a U-shaped association. No association was found between coffee consumption and cancer mortality in men, whereas a weak inverse association was found in women. Sequent exclusion of deaths that occurred within 2–8 years from baseline did not alter the overall results; among women, however, the reduction in total cancer mortality risk was weakened and was not significant.

Previous cohort studies examined coffee consumption in relation to all-cause mortality, and the results were controversial. Early studies tended to find direct associations [6, 7]. However, recently, the occurrence of inflammation or coronary heart disease were found to be inversely associated with coffee consumption by some cohort studies [4, 9], and an inverse [3, 5] or U-shaped [8, 20] association between coffee consumption and all-cause mortality has also been reported. The results of our study detecting an inverse association among men and a U-shaped association among women were in line with these recent studies.

Some cohort studies have also examined the effect of coffee consumption on total cancer mortality. Most of these studies found no association [3, 4, 6, 9] while two studies found an inverse but not significant association [5, 11]. Our study also found no association among men and a weak inverse association among women between coffee consumption and total cancer mortality, even after exclusion of those with a past history of cancer, MI or stroke. Although the weak inverse association among women disappeared with the exclusion of deaths that occurred within 6 years after baseline, it suggests that at the least, there are no harmful effects of coffee on total cancer mortality.

Inconsistent results among men between the risks of all-cause mortality and total cancer mortality in our study must be due to an association with other causes of death, as cancer deaths accounted for about one-third of total mortality. We previously reported an inverse association between coffee consumption and the risk of total cardiovascular disease mortality among men [21]. In addition, the incidence of diabetes mellitus was found to be lower with increasing coffee consumption [22]. Some recent cohort

Table 3 Hazard ratios for all-cause and total cancer mortality by coffee consumption stratified by age group at baseline

	Men					Women				
	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	Trend *P*	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	Trend *P*
Person-years										
Aged 40–59 years at baseline	231,833	39,599	62,216	12,682		327,339	70,973	76,533	9,599	
Aged 60–79 years at baseline	181,677	22,314	21,585	2,863		286,268	40,493	24,340	1,345	
All causes										
Aged 40–59 years at baseline										
Cases	2,097	250	366	63		1,204	214	177	25	
Age-adjusted HR (95% CI)	1.00	0.78 (0.68–0.89)	0.83 (0.74–0.93)	0.79 (0.61–1.01)	<.001	1.00	0.94 (0.82–1.09)	0.82 (0.70–0.96)	1.04 (0.70–1.56)	0.07
Multivariable-adjusted HR (95% CI)	1.00	0.80 (0.70–0.91)	0.80 (0.71–0.90)	0.69 (0.54–0.89)	<.0001	1.00	0.94 (0.81–1.10)	0.79 (0.67–0.93)	0.83 (0.56–1.25)	0.01
Aged 60–79 years at baseline										
Cases	6,892	785	638	87		5,894	524	297	19	
Age-adjusted HR (95% CI)	1.00	1.00 (0.93–1.08)	0.93 (0.86–1.01)	0.96 (0.78–1.19)	0.35	1.00	0.77 (0.70–0.84)	0.85 (0.75–0.95)	1.02 (0.65–1.60)	<.0001
Multivariable-adjusted HR (95% CI)	1.00	1.01 (0.93–1.08)	0.90 (0.83–0.98)	0.89 (0.72–1.10)	0.06	1.00	0.79 (0.72–0.86)	0.86 (0.76–0.97)	0.88 (0.56–1.39)	<.0001
Total cancer										
Aged 40–59 years at baseline										
Cases	943	107	175	43		584	111	98	10	
Age-adjusted HR (95% CI)	1.00	0.75 (0.61–0.92)	0.91 (0.77–1.07)	1.25 (0.92–1.70)	0.96	1.00	1.00 (0.81–1.22)	0.91 (0.73–1.13)	0.84 (0.45–1.57)	0.35
Multivariable-adjusted HR (95% CI)	1.00	0.77 (0.63–0.95)	0.90 (0.76–1.06)	1.16 (0.84–1.59)	0.77	1.00	0.99 (0.80–1.22)	0.89 (0.71–1.11)	0.71 (0.38–1.35)	0.17
Aged 60–79 years at baseline										
Cases	2,303	301	261	33		1,558	167	93	7	
Age-adjusted HR (95% CI)	1.00	1.11 (0.99–1.26)	1.05 (0.93–1.20)	1.01 (0.72–1.43)	0.14	1.00	0.84 (0.71–0.98)	0.84 (0.68–1.04)	1.17 (0.56–2.47)	0.05
Multivariable-adjusted HR (95% CI)	1.00	1.14 (1.01–1.29)	1.03 (0.90–1.17)	0.94 (0.67–1.33)	0.40	1.00	0.83 (0.70–0.98)	0.82 (0.66–1.02)	1.00 (0.47–2.12)	0.03

Multivariable-adjusted HR; adjusted for age categories, smoking status, alcohol drinking, walking hours, sleep duration, body mass index, consumption of green-leafy vegetables, green tea consumption, education, stress, marital status, past history of cancer, myocardial infarction or stroke

Table 4 Multivariable-adjusted hazard ratios of all-cause and total cancer mortality by coffee consumption with exclusion of subjects with past disease history or early deaths

	Men					Women				
	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	Trend *P*	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	Trend *P*
Person-years										
Past history of cancer, MI or stroke excluded	394,503	59,430	81,228	15,280		586,564	106,547	97,900	10,586	
Death within first 2 years excluded	412,686	61,811	83,653	15,516		612,881	111,349	100,765	10,934	
Death within first 4 years excluded	409,867	61,373	83,212	15,434		610,371	111,043	100,436	10,907	
Death within first 6 years excluded	404,285	60,619	82,333	15,246		605,595	110,290	99,993	10,820	
Death within first 8 years excluded	395,733	59,535	80,980	15,077		597,756	109,188	99,125	10,722	
All cause										
Past history of cancer, MI or stroke excluded										
Cases	8,102	933	910	142		6,413	675	434	43	
HR (95% CI)	1.00	0.94 (0.88–1.01)	0.85 (0.79–0.91)	0.79 (0.67–0.94)	<.0001	1.00	0.83 (0.77–0.91)	0.83 (0.75–0.92)	0.94 (0.69–1.27)	<.0001
Death within first 2 years excluded										
Cases	8,382	972	940	143		6,727	689	446	43	
HR (95% CI)	1.00	0.96 (0.90–1.03)	0.87 (0.82–0.94)	0.83 (0.70–0.98)	<.001	1.00	0.82 (0.75–0.89)	0.83 (0.75–0.92)	0.92 (0.68–1.25)	<.0001
Death within first 4 years excluded										
Cases	7,606	864	848	130		6,183	637	405	42	
HR (95% CI)	1.00	0.96 (0.89–1.03)	0.87 (0.81–0.94)	0.82 (0.69–0.98)	<.001	1.00	0.84 (0.77–0.91)	0.84 (0.75–0.93)	1.00 (0.73–1.36)	<.001
Death within first 6 years excluded										
Cases	6,653	744	729	106		5,512	555	359	37	
HR (95% CI)	1.00	0.96 (0.89–1.04)	0.88 (0.81–0.95)	0.77 (0.64–0.94)	<.001	1.00	0.84 (0.77–0.92)	0.85 (0.76–0.95)	1.00 (0.72–1.39)	<.01
Death within first 8 years excluded										
Cases	5,568	606	594	93		4,673	450	296	31	
HR (95% CI)	1.00	0.97 (0.89–1.06)	0.88 (0.80–0.96)	0.82 (0.67–1.01)	<.01	1.00	0.86 (0.78–0.95)	0.88 (0.78–0.99)	1.04 (0.73–1.49)	0.03
Cancer										
Past history of cancer, MI or stroke excluded										
Cases	3,039	378	401	74		1,983	254	177	17	
HR (95% CI)	1.00	1.01 (0.90–1.12)	0.94 (0.85–1.05)	1.06 (0.84–1.35)	0.98	1.00	0.88 (0.77–1.01)	0.84 (0.72–0.99)	0.89 (0.55–1.44)	0.02
Death within first 2 years excluded										
Cases	3,034	382	411	75		2,001	258	183	17	
HR (95% CI)	1.00	1.03 (0.92–1.15)	0.99 (0.88–1.10)	1.12 (0.89–1.42)	0.38	1.00	0.88 (0.77–1.01)	0.87 (0.74–1.02)	0.86 (0.53–1.40)	0.04
Death within first 4 years excluded										
Cases	2,728	335	373	69		1,798	233	161	16	
HR (95% CI)	1.00	1.01 (0.90–1.13)	0.99 (0.88–1.11)	1.13 (0.88–1.44)	0.34	1.00	0.90 (0.78–1.03)	0.85 (0.72–1.01)	0.90 (0.55–1.49)	0.05
Death within first 6 years excluded										
Cases	2,335	290	321	49		1,552	196	142	14	
HR (95% CI)	1.00	1.04 (0.91–1.17)	1.00 (0.88–1.13)	0.93 (0.70–1.24)	0.93	1.00	0.90 (0.77–1.05)	0.89 (0.74–1.06)	0.92 (0.54–1.57)	0.14

Table 4 continued

	Men					Women				
	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	Trend P	<1 cup/day	1 cup/day	2–3 cups/day	4+ cups/day	Trend P
Death within first 8 years excluded										
Cases	1,922	235	257	42		1,271	154	116	12	
HR (95% CI)	1.00	1.05 (0.91–1.21)	0.99 (0.86–1.13)	0.97 (0.71–1.32)	0.96	1.00	0.91 (0.77–1.08)	0.92 (0.76–1.13)	1.00 (0.56–1.79)	0.46

HR adjusted for age categories, smoking status, alcohol drinking, walking hours, sleep duration, body mass index, consumption of green-leafy vegetables, green tea consumption, education, stress, marital status, past history of cancer, myocardial infarction or stroke

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studies examined the risk of coffee consumption on cardiovascular diseases, which was also an inverse association [4, 9]. Cardiovascular deaths accounted for 28.3% of men's deaths in our study; thus, this may be the main reason that an inverse association with all-cause mortality occurs, in spite of no association with total cancer mortality among men.

Recently, coffee has been found to be one of the major sources of antioxidants in the diet [23] and has beneficial effects on inflammation [9]. It has been found that plasma antioxidants increased [24] and biomarkers of oxidative stress decreased [25] after coffee intake. Prolonged inflammation may contribute not only to atherosclerosis and ischemic heart diseases, but also to cancer. The beneficial effects of coffee on all-cause mortality and total cancer mortality might be understandable from this point of view. On the other hand, there is a possibility that possible subclinical diseases lead to a reduction in coffee consumption. In fact, those in the lowest coffee consumption group had more past disease history than those who consumed 1 cup of coffee a day or more in our study. This poorer health condition might be linked to a risk elevation among non-coffee drinkers. However, analyses to examine the reverse causation with sequent exclusion of deaths that occurred within 2–8 years from baseline did not alter the relationship between coffee consumption and all-cause mortality, suggesting that the difference in health at baseline was not the only explanation of the associations that were found.

There are two methodological limitations in this study. First, we obtained information only at baseline. Moreover, coffee consumption was estimated from self-report. Thus, some measurement errors at baseline were inevitable. Second, detailed data on coffee consumption, such as choice of caffeinated or decaffeinated and methods of brewing, were not collected. The methods of coffee preparation and habits of coffee drinking may change considerably with time and vary geographically. Therefore, other cohort studies with detailed coffee consumption data are required.

In conclusion, the present cohort study suggested the beneficial effects of coffee consumption on all-cause mortality among both men and women. In addition, the results indicated no association between coffee consumption and total cancer mortality among men and a weak inverse association among women, suggesting that no harmful effects of coffee on total cancer mortality.

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Conflict of interest The authors declare that there are no conflicts of interest.

Appendix: Member list of the JACC study group

The present members of the JACC Study Group who coauthored this paper together with their affiliations are as follows: Dr. Akiko Tamakoshi (present chairperson of the study group), Aichi Medical University School of Medicine; Drs. Mitsuru Mori & Fumio Sakauchi, Sapporo Medical University School of Medicine; Dr. Yutaka Motohashi, Akita University School of Medicine; Dr. Ichiro Tsuji, Tohoku University Graduate School of Medicine; Dr. Yosikazu Nakamura, Jichi Medical School; Dr. Hiroyasu Iso, Osaka University School of Medicine; Dr. Haruo Mikami, Chiba Cancer Center; Dr. Michiko Kurosawa, Juntendo University School of Medicine; Dr. Yoshiharu Hoshiyama, University of Human Arts and Sciences; Dr. Naohito Tanabe, Niigata University School of Medicine; Dr. Koji Tamakoshi, Nagoya University Graduate School of Health Science; Dr. Kenji Wakai, Nagoya University Graduate School of Medicine; Dr. Shinkan Tokudome, National Institute of Health and Nutrition; Dr. Koji Suzuki, Fujita Health University School of Health Sciences; Dr. Shuji Hashimoto, Fujita Health University School of Medicine; Dr. Shogo Kikuchi, Aichi Medical University School of Medicine; Dr. Yasuhiko Wada, Faculty of Human Life and Environmental Science, Kochi Women's University; Dr. Takashi Kawamura, Kyoto University Center for Student Health; Dr. Yoshiyuki Watanabe, Kyoto Prefectural University of Medicine Graduate School of Medical Science; Dr. Kotaro Ozasa, Radiation Effects Research Foundation; Dr. Tsuneharu Miki, Kyoto Prefectural University of Medicine Graduate School of Medical Science; Dr. Chigusa Date, School of Human Science and Environment, University of Hyogo; Dr. Kiyomi Sakata, Iwate Medical University; Dr. Yoichi Kurozawa, Tottori University Faculty of Medicine; Drs. Takesumi Yoshimura & Yoshihisa Fujino, University of Occupational and Environmental Health; Dr. Akira Shibata, Kyushu University Institute of Health Science; Dr. Naoyuki Okamoto, Kanagawa Cancer Center; and Dr. Hideo Shio, Moriyama Municipal Hospital.

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Number of children and all-cause mortality risk: results from the Japan Collaborative Cohort Study

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Background: The mean total birth rate of the world had been gradually decreasing, with the rate in Japan now at its lowest level internationally. From a public health perspective, it is important to determine the impact of the number of children on all-cause mortality. **Methods:** A total of 96 311 individuals from the Japan Collaborative Cohort Study were followed from 1988–90 for an average of 14.4 years. Hazard ratios (HRs) with a 95% confidence interval were calculated from proportional hazard models to estimate the risk of all-cause mortality according to the number of children. **Results:** As of 2006, a total of 18 807 deaths had occurred. Both childless men and women showed higher all-cause mortality risks than those with two children (HR: 1.17 in men and 1.29 in women). Those with one child also showed higher risks (1.13 and 1.16, respectively). Having four or more children among men and five or more children among women also posed a risk (1.16 in men with four children and 1.22 in women with five or more children), showing a U-shaped association between the number of children and all-cause mortality risk. The risk of having only one child seemed evident with the decrease in age among both men and women, while the risk of having many children was apparent with the increase in age. **Conclusion:** We found a U-shaped association between the number of children and all-cause mortality among both men and women, with the lowest risk among those with two children.

Keywords: childless, cohort study, mortality, number of children

Introduction

Because of the possible biological effects of reproductive history on mortality, many studies have examined the relationship between the number of children and all-cause mortality among women. However, only a few studies have focused on both men and women.[1–5] Norwegian census data covering 14.5 million person-years revealed that, compared to parents with two children, those who were childless or had only one child showed a significantly elevated risks of all-cause mortality, whereas those with three or more children showed significantly decreased risks in both men and women.[2] In rural Bangladesh, where the mean number of parity was seven live births at the time of the study, Hurt *et al.*[3] reported no association between parity and long-term mortality among women; however, there was a small but significant decrease in mortality among men with an increasing number of live births. A cohort study of those born between 1880 and 1929 found that parous women had a significantly poorer survival rate than nulliparous women (especially among those born earlier), but not among men.[1] These inconsistent findings among different periods, countries and cultures suggest that some social factors might be related to the association between the numbers of children and all-cause mortality risks.

According to the Population Database announced by the United Nations,[6] the mean total fertility rate of the world had been gradually decreasing (4.92 in 1950–55, 4.32 in 1970–75 and 2.67 in 2000–05), and is predicted to fall to 2.02 by 2045–50. In Japan, birth rates have been dramatically decreasing in recent years, with the total fertility rate now at its lowest level internationally (1.37 in 2008). Since such a dramatic decrease in the fertility rate is occurring worldwide, from a public health perspective, it is important to determine the impact of the number of children on all-cause mortality among both men and women in Japan, a country with one of the greatest decreases in this regard. In this study, using a large-scale cohort study initiated in Japan ~20 years ago, we investigated the relationship between the number of children and all-cause mortality among Japanese adults.

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Methods

Subjects and data collection

The Japan Collaborative Cohort Study (JACC Study) was conducted from 1988 to 1990, enrolling healthy subjects living in 45 areas of Japan, and collecting baseline data using a self-administered questionnaire. Sampling methods and detailed protocols have been published elsewhere.[7,8] A total of 110 792 subjects (46 465 males and 64 327 females) aged 40–79 years participated in the study.

Information about their children was obtained by self-administered questionnaires. Subjects were grouped into six categories according to the numbers of children—those who were childless, and those with 1, 2, 3, 4 and ≥5 children. A total of 96 311 individuals (40 073 men and 56 238 women) who provided valid responses were regarded as eligible for the analysis.

Our entire study design was approved by the Ethical Board of the Nagoya University School of Medicine, where the central secretariat of the JACC Study was located.

Follow-up

The causes and dates of death among the subjects were identified by reviewing all death certificates in each area with the permission of the Director-General of the Prime Minister's Office (Ministry of Internal Affairs and Communications). Those who moved out of a study area were treated as censored, because deaths after such moves could not be confirmed by our follow-up system. We followed the subjects until the end of 2006, except in eight areas where follow-ups were discontinued at the end of 1999 or 2003.

Analysis

The distribution of some socio-demographic factors that were known to be risk factors for all-cause mortality among groups of numbers of children were compared using an analysis of variance or the Mantel–Haenszel test adjusted for age. Hazard ratios (HRs) and 95% confidence interval (CI) were calculated using the Cox's proportional hazard model adjusted for 5-year age groups stratified by gender. We drew a graph of the log(−log(survival)) vs. log of survival time to check the proportional hazard assumption between the number of children. The lines were approximately parallel, confirming that the assumption was suitable. In multivariate analyses, we further adjusted several factors associated with all-cause mortality and/or number of children, as well as the subjects' residential area grouped into seven by geographic location—that is, smoking status (current smoker, quitter or never-smoker), drinking status (current drinker, quitter or never-drinker), daily walking length (walking >1 h per day or not), sleep length (<6.5 h, 6.5–8.4 h, 8.5 h or longer), consumption of green-leafy vegetables (almost daily or not), perceived stress (yes or no), education (attended school up to 15-years old, 18-years old or older), body mass index (BMI < 18.5, 18.5–24.9, 25.0≤), occupational status (employed, self-employed or not), marital status (having a spouse or not), disease history (of cancer, cardiovascular disease or stroke or none), all of these data were obtained from self-administered questionnaires. For all covariates, missing values were treated as an additional category in the model. Further analysis stratified by baseline age (10 years each) in addition to gender was also conducted. All statistical analyses were performed using the Statistical Analysis System (SAS 9.1, Cary, NC, USA) at the Aichi Medical University Computer Center.

Results

During the average follow-up period of 14.4 years, a total of 4733 movements (1732 men and 3001 women) and 18 807 deaths occurred (10 849 men and 7958 women). Total cancer deaths accounted for 37.1% in men and 31.3% in women, with circulatory system deaths accounting for 28.6% and 34.6%, respectively.

A total of 1369 (3.4%) men and 2026 (3.6%) women were childless at baseline. The largest group was composed of those with two children among both men (41.6%) and women (38.2%), followed by those with three children (32.7% in men and 30.8% in women) (table 1). Compared to those with one child, childless subjects were older, less likely to have a spouse, to walk, to eat green-leafy vegetables, or to show a healthy BMI (18.5–24.9), and were more likely to suffer from mental stress, to have a history of cancer, cardiovascular disease or stroke, less likely to be employed among both men and women, and more likely to be non-drinkers among men. Among subjects with children, those with two children were the youngest (54.9 years among men and 54.1 years among women), with the mean age increasing according to the increasing number of children, and the oldest mean age found among those with five or more children (69.9 among men and 69.7 among women). The proportion of those having a spouse, sleeping 6.5–8.4 h per night, with a BMI of 18.5–24.9, being highly educated, without a history of cancer, cardiovascular disease or stroke and employed showed a similar pattern, reaching its highest among those with two children and its lowest among those with five or more in both men and women.

The all-cause mortality risks according to the number of children are shown in table 2. Compared to those with two children, the childless group and that with one child showed a slightly higher all-cause mortality risk—1.31 (95% CI 1.19–1.45) in men and 1.37 (1.24–1.53) in childless women and 1.18 (1.10–1.28) in men and 1.21 (1.10–1.32) in women with one child; though those values decreased slightly after adjusting for potential risk factors, they still remained at statistically significant levels. Men with three children showed no risk elevation, whereas men with four or more children were associated with elevated risk. A similar trend was observed among women, although the protective effect of having children was more apparent than in men, and only those with five or more children showed an excessive risk.

With stratification by age group at the baseline, different distribution patterns of the number of children were observed. Among those aged 40–49 years, most had two children (51.5% men and 52.6% women). However, the proportion was diminished according to increasing age, and among those aged 70–79 years, these values declined to 16.1 and 13.1%, respectively. In contrast, those with five or more children were rare among the younger generation (0.6 and 0.6% among men and women aged 40–49 years) increasing according to increasing age and reaching 20.3 and 31.5% among those aged 70–79 years. As shown in table 3, among both men and women, the risk of having only one child became apparent with age reduction, though the risk among men aged 40–49 years was lower than that in other age groups. Moreover, all-cause mortality risks of the childless were lower than those with one child among them: 1.19 (0.78–1.81) and 1.05 (0.79–1.40) among men aged 40–49, 1.36 (0.79–2.32) and 1.53 (1.13–2.09) among women aged 40–49 years and 0.89 (0.63–1.25) and 1.29 (1.07–1.57) among women aged 50–59, respectively, while, the values were 1.13 (0.95–1.34) and 1.08 (0.92–1.27) among men aged 70–79 and 1.36 (1.15–1.62) and 1.09 (0.93–1.27) among women aged 70–79, respectively. In contrast, the risk of having many children was evident from the age increment. Especially among women, null associations

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Table 1 Distribution of some demographic factors according to the number of children: baseline of the JACC Study 1988–90, Japan

	Men						Women					
	0	1	2	3	4	≥5	0	1	2	3	4	≥5
Number	1369	3159	16 656	13 110	3875	1904	2026	4842	21 508	17 348	6228	4286
Age (years)	58.1 ± 11.0	56.5 ± 9.9	54.9 ± 8.9	57.8 ± 9.9	64.9 ± 9.6	69.9 ± 8.0***	60.6 ± 10.1	57.6 ± 9.8	54.1 ± 8.7	57.1 ± 9.5	63.9 ± 8.8	69.7 ± 7.3***
Having a spouse	668 (48.8)	2632 (83.3)	14 540 (87.3)	11 208 (85.5)	3052 (78.8)	1391 (73.1)***	916 (45.2)	3254 (67.2)	17 059 (79.3)	13 234 (76.3)	4069 (65.3)	2397 (55.9)***
Never-smoker	256 (18.7)	567 (17.9)	3340 (20.1)	2585 (19.7)	684 (17.7)	372 (19.5)*	1522 (75.1)	3712 (76.7)	17 690 (82.2)	14 470 (83.4)	5013 (80.5)	3384 (79.0)***
Non-drinker	336 (24.5)	606 (19.2)	2951 (17.7)	2162 (16.5)	742 (19.1)	426 (22.4)***	1345 (66.4)	3236 (66.8)	14 665 (68.2)	12 111 (69.8)	4365 (70.1)	3068 (71.6)*
Walking ≥ 1 h day^{-1}	358 (26.2)	1225 (38.8)	6935 (41.6)	5535 (42.2)	1646 (42.5)	771 (40.5)***	561 (27.7)	1953 (40.3)	9215 (42.8)	7652(44.1)	2740 (44.0)	1704 (39.8)***
Sleep 6.5–8.4 h per night	840 (61.4)	2198 (69.6)	11 906 (71.5)	9088 (69.3)	2402 (62.0)	1054 (55.4)*	1234 (60.9)	3101 (64.0)	14 090 (65.5)	11 154 (64.3)	3732 (59.9)	2353 (54.9)***
Eating green-leafy vegetables almost daily	215 (15.7)	771 (24.4)	4259 (25.6)	3501 (26.7)	1132 (29.2)	541 (28.4)***	469 (23.1)	1497 (30.9)	6665 (31.0)	5278 (30.4)	1995 (32.0)	1275 (29.7)
BMI 18.5–24.9	922 (67.3)	2278 (72.1)	12 339 (74.1)	9559 (72.9)	2731 (70.5)	1284 (67.4)***	1204 (59.4)	3227 (66.6)	15 091 (70.2)	11 750 (67.7)	3907 (62.7)	2426 (56.6)***
College or higher education	180 (13.1)	459 (14.5)	2554 (15.3)	1767 (13.5)	473 (12.2)	184 (9.7)***	200 (9.9)	430 (8.9)	2073 (9.6)	1302 (7.5)	316 (5.1)	139 (3.2)***
Low mental stress	581 (42.4)	1898 (60.1)	9910 (59.5)	7731 (59.0)	2387 (61.6)	1124 (59.0)	954 (47.1)	2983 (61.6)	13 642 (63.4)	10 918 (62.9)	3954 (63.5)	2607 (60.8)***
Without a history of cancer, cardiovascular disease or stroke	869 (63.5)	2326 (73.6)	12 774 (76.7)	9834 (75.0)	2651 (68.4)	1247 (65.5)	1366 (67.4)	3437 (71.0)	16 192 (75.3)	12 986 (74.9)	4383 (70.4)	2899 (67.6)***
Employed	410 (29.9)	1259 (39.9)	7143 (42.9)	4028 (30.7)	617 (15.9)	176 (9.2)***	335 (16.5)	1079 (22.3)	5907 (27.5)	3516 (20.3)	625 (10.0)	226 (5.3)

Data represent number of individuals (percentage in parentheses) or mean ± standard error for continuous variables

*P < 0.05, **P < 0.01, ***P < 0.001 performed by ANOVA for age or performed by Mantel–Haenszel test adjusted for age (categorical data)

Table 2 All-cause mortality risks according to the number of children: the JACC Study 1988–2006, Japan

	Men						Women					
	0	1	2	3	4	≥5	0	1	2	3	4	≥5
Total												
Person-years	19 118	44 364	244 999	188 060	48 944	21 202	29 334	68 648	323 455	259 853	88 158	55 328
Cases	474	799	3205	3449	1765	1157	469	685	1783	2032	1342	1647
Age-adjusted HR												
HR (95% CI)	1.31 (1.19–1.45)	1.18 (1.10–1.28)	1.00	1.05 (1.00–1.10)	1.20 (1.13–1.28)	1.32 (1.23–1.42)	1.37 (1.24–1.53)	1.21 (1.10–1.32)	1.00	1.02 (0.96–1.09)	1.07 (0.99–1.15)	1.24 (1.15–1.34)
Multi-adjusted HR												
HR (95% CI)	1.17 (1.06–1.30)	1.13 (1.04–1.22)	1.00	1.04 (0.99–1.09)	1.16 (1.09–1.24)	1.25 (1.16–1.35)	1.29 (1.16–1.44)	1.16 (1.06–1.27)	1.00	1.03 (0.97–1.10)	1.06 (0.99–1.15)	1.22 (1.13–1.32)

Multi-adjusted HRs were adjusted for age, residential area group, marital status, smoking status, alcohol consumption status, walking hours, sleeping hours, consuming green-leafy vegetables, BMI, education, mental stress, disease history and employment status

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Table 3 All-cause mortality risks stratified by age categories according to the number of children: the JACC Study 1988–2006, Japan

	Men						Women					
	0	1	2	3	4	≥5	0	1	2	3	4	≥5
Aged 40–49 at baseline (years)												
Person-years	5435	13 666	81 418	50 963	5578	936	5417	17 709	112 303	69 195	8063	1292
Cases	30	58	304	197	26	4	16	53	192	127	10	2
HR (95% CI)	1.19 (0.78–1.81)	1.05 (0.79–1.40)	1.00	1.02 (0.85–1.22)	1.20 (0.80–1.80)	0.96 (0.35–2.58)	1.36 (0.79–2.32)	1.53 (1.13–2.09)	1.00	1.05 (0.84–1.32)	0.67 (0.35–1.27)	0.78 (0.19–3.13)
Aged 50–59 at baseline (years)												
Person-years	6108	15 188	93 474	58 545	8320	2136	8833	24426	131 009	86 419	16 678	4433
Cases	89	198	955	638	120	24	38	136	518	361	72	21
HR (95% CI)	1.20 (0.95–1.51)	1.24 (1.06–1.45)	1.00	1.07 (0.97–1.19)	1.34 (1.10–1.62)	0.91 (0.60–1.36)	0.89 (0.63–1.25)	1.29 (1.07–1.57)	1.00	1.04 (0.91–1.19)	1.02 (0.80–1.32)	1.01 (0.65–1.58)
Aged 60–69 at baseline (years)												
Person-years	4978	12 147	60 022	60 544	20 368	6635	9815	19 175	67 876	85 256	42 622	20 312
Cases	165	328	1398	1564	643	249	165	237	694	905	530	317
HR (95% CI)	1.17 (0.99–1.39)	1.09 (0.97–1.23)	1.00	1.02 (0.95–1.10)	1.18 (1.08–1.30)	1.35 (1.18–1.55)	1.31 (1.09–1.56)	1.11 (0.95–1.28)	1.00	0.98 (0.89–1.08)	1.05 (0.94–1.18)	1.24 (1.08–1.42)
Aged 70–79 at baseline (years)												
Person-years	2597	3364	10 085	18 008	14 678	11 495	5268	7338	12 268	18 984	20 795	29 291
Cases	190	215	548	1050	976	880	250	259	379	639	730	1307
HR (95% CI)	1.13 (0.95–1.34)	1.08 (0.92–1.27)	1.00	1.04 (0.94–1.16)	1.11 (1.00–1.24)	1.22 (1.09–1.37)	1.36 (1.15–1.62)	1.09 (0.93–1.27)	1.00	1.12 (0.98–1.27)	1.09 (0.96–1.23)	1.23 (1.09–1.38)

Multi-adjusted HRs were adjusted for age, residential area group, marital status, smoking status, alcohol consumption status, walking hours, sleeping hours, consuming green-leafy vegetables, BMI, education, mental stress, disease history and employment status

between having many children and all-cause mortality were found in those aged 40–49 and 50–59 years; however, in women aged 60–69 and 70–79 years, the risks of having five or more children were 1.24 (1.08–1.42) and 1.23 (1.09–1.38), respectively, compared with those having two children.

Discussion

Using the large data set of a population-based cohort study, we found that both men and women with no or only one child were at a significantly higher risk of all-cause mortality compared with those with two children. Additionally, men with four or more children and women with five or more children also had a significantly high risk, showing the U-shaped association with the number of children, with its lowest risk at having two children. The risk of having only one child seemed evident with the decrease in age among both men and women, while the risk of having many children was particularly apparent with the increase in age.

Results of studies examining the relationship between the number of children and all-cause mortality varied. A study conducted in Bangladesh showing that survival for both sexes was greatly enhanced by an increasing number of surviving children, regardless of parity or other social factors, indicates the social and economic advantages of having children in developing countries.[3] A study from Norway—where child allowances are relatively large and many 'family-friendly' policies exist—also found no high-parity disadvantage, suggesting that there are various health benefits of having several children in such an environment that may outweigh the costs.[2] Meanwhile, an Israeli study based on census data recently found a U-shaped association between the number of children and all-cause mortality, with the lowest risk being evident among those with 3–4 children for both sexes.[4] There were only a few studies examining the relationship among both men and women; however, among women, some other studies have also reported a U-shaped association with women with two children experiencing the lowest risk, as in our present study.[9–11] Such inconsistent results can be expected, since the impact of having children on the subjects' health might be considerably modified by their surrounding social factors.

The existence of children may provide subjects with parental roles, family obligations and social networks through childbearing.[5] These social factors may be related to healthier behavior,[2] may increase well-being,[12] and may consequently affect subsequent survival. Moreover, adult children may provide support to their elderly parents, mediate social services and monitor their health behavior.[5] Such conditions may offer a positive effect of having children, and subsequently lower the all-cause mortality risk. Although large family size usually involves parents in physical activity, it might also lead to excessive physical and mental stress.[13] Moreover, those with many children tended to start their parenthood earlier, with early parenthood known to be associated with poor health.[2] These social factors might partly explain the disadvantage in all-cause mortality risk of having many children. On the other hand, among the childless, especially among the elderly, the absence of social support from children might lead to an increase in all-cause mortality risk.[5]

The disadvantage of having no children was more apparent in women than in men, while the disadvantage of having many children was less in women than in men. Among women, the association between the number of children and all-cause mortality may also involve a biological mechanism, such as hormonal protection.[14,15] A prospective study of Norwegian women found inverse associations with parity and cancers of the breast, uterus and ovary.[16] Using the Swedish registry

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system, Mogren *et al.*[17] also revealed that multiparity was a protective factor for all gynecological cancers, including cervical and breast cancers. We also previously demonstrated the protective effects of having children against the development of colon cancer among women.[15] Such effects on hormone-related diseases might be related to the biological changes initiated by conception and that lead to parity and breastfeeding, and might explain the somewhat different impact between men and women of having many children. On the other hand, parous women had a higher mortality rate from diabetes mellitus, ischemic heart disease and cerebrovascular disease than nulliparous women.[18] Parity was found to increase the risk of atherosclerosis through its effect on lower high-density lipoprotein levels and higher glucose/insulin ratios.[19] The negative effect on cardiovascular disease may explain the elevated mortality risk in women with five or more children.

Impacts on all-cause mortality differed with the number of children in the different age categories in our results, particularly among women. All-cause mortality is comprised of a combination of death from cancer, cardiovascular disease and other diseases, and the combination of causes of death differs with age. In fact, in our data set, the proportion of cancer deaths diminished with age (from 42.7 and 52.9% of total deaths in men and women aged 40–49 years to 26.9 and 21.1% in those aged 70–79 years, respectively), while cardiovascular deaths increased (from 19.8 and 19.5% up to 32.7 and 40.9%, respectively). This different composition might partially explain the differences in risk among different age categories. Some previous studies have examined the association between the number of children and all-cause mortality risks by age categories. The younger the women, the greater was the all-cause mortality risk of being childless, as was found in England, Wales, Austria[9] and Norway.[2] One study reported that the risk of all-cause mortality with many children was higher in those aged 50 years and older than in those aged <50 years.[16] Grundy and Tomassini, however, found the opposite result (all-cause mortality risk with five or more children was higher in a younger cohort than in the elderly).[11] The reason for such an inconsistent finding was unclear, but because of the availability of contraception and legal abortion, having many children might be based on one's choice among more recent generations than among earlier ones.[2] In fact, our results showed that there was a lower risk of many children among the middle-aged group than in the elderly group. In addition, from our study, the background of those without children and those with one child might be differ in the middle-aged and elderly group, though the middle-aged involved fewer events, given that they were less likely to die than the elderly. Mortality risks among those with one child were higher than those without children among middle-aged women and men except men aged 40–49 years unlikely to elder groups. This might be because the childless were better educated, more financially secure, and might feel more in control of their lives than women with children do nowadays.[20] At least we could say that not only the different causes of death in each age category but also different social and environmental factors between elderly and middle-aged women's cohorts might affect the association between the number of children and all-cause mortality risks.

The strong points of our study are as follows: (i) a large-scale cohort with more than 90 000 subjects from all over Japan; (ii) a long follow-up period of ~14.4 years; (iii) multiple lifestyle variables collected at baseline and (iv) adjusting as much as possible for potential confounders. Such advantages allowed us to separately estimate the association between the number of children and all-cause mortality by baseline age, while adjusting for various factors.

One limitation in this study was that the underlying reasons for being childless remain unknown. One possible reason for the high mortality-risk of childless women might be the poor health of our subjects. Childless subjects in our study were physically inactive, had an unhealthy BMI (<18.5 or ≤25), suffered from a high level of mental stress, and had a history of cancer, cardiovascular disease or stroke, compared to their counterparts with children. This suggests the possibility that the elevated risk among the childless group was due to neither the result of a reduced social network nor to other possible mechanisms, but simply to poor health. According to the statistics, the total fertility rate has rapidly decreased in Japan. It was around 2.0 from 1957 to 1974, but has fallen to below 1.50 since 1993.[21] The proportion of childless Japanese couples after 15–19 continuous years of marriage increased from 3.0% in 1977 to 5.6% in 2005.[22] Moreover, not only in Japan but also globally, fertility rates are declining.[6] Even if the main reason for being childless in the past was poor health, it may now be changing, so that assumptions cannot easily be made about the mortality risk patterns of childless men and women. Another limitation was that, although we had adjusted for potential confounding factors such as smoking status and physical activity, there was still a possibility that other factors (particularly social or psychological factors) might have confounded our findings. A large-scale cohort study with information on such social factors will be required to effectively investigate the true relationship between the number of children and mortality.

In conclusion, we found a U-shaped association between the number of children and all-cause mortality among both men and women, with the lowest risk among those with two children. Our main finding was that having no children or only one child may lead to an elevated risk of total mortality in view of the decrease in total future fertility rates, while emphasizing that a high risk in men and women with many children may not play an important role in the decline of fertility rates.

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Conflicts of interest: None declared.

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Key points

- The mean total birth rate worldwide has been gradually decreasing, with the rate in Japan now at its lowest level internationally.
- We found that being childless or having only one child may lead to an elevated risk of total mortality.
- Men with four or more children and women with five or more children also showed a high risk of all-cause mortality.

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Appendix 1

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Review

In Vitro and *In Vivo* Genotoxicity Induced by Fullerene (C_{60}) and Kaolin

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Nanomaterials are being utilized for many kinds of industrial products, and the assessment of genotoxicity and safety of nanomaterials is therefore of concern. In the present study, we examined the genotoxic effects of fullerene (C_{60}) and kaolin using *in vitro* and *in vivo* genotoxicity systems. Both nanomaterials significantly induced micronuclei and enhanced frequency of sister chromatid exchange (SCE) in cultured mammalian cells. When ICR mice were intratracheally instilled with these nanomaterials, DNA damage of the lungs increased significantly that of the vehicle control. Formation of DNA adducts in the lungs of mice exposed to nanomaterials were also analyzed by stable isotope dilution LC-MS/MS. 8-Oxodeoxyguanosine and other lipid peroxide related adducts were increased by 2- to 5-fold in the nanomaterial-exposed mice. Moreover, multiple (four consecutive doses of 0.2 mg per animal per week) instillations of C_{60} or kaolin, increased *gpt* mutant frequencies in the lungs of *gpt* delta transgenic mice. As the result of mutation spectrum analysis, G:C to C:G transversions were commonly increased in the lungs of mice exposed to both nanomaterials. In addition, G:C to A:T was increased in kaolin-exposed mice. In immunohistochemical analysis, many regions of the lungs that stained positively for nitrotyrosine (NT) were observed in mice exposed to nanomaterials. From these observations, it is suggested that oxidative stress and inflammatory responses are probably involved in the genotoxicity induced by C_{60} and kaolin.

Key words: nanomaterials, genotoxicity, fullerene (C_{60}), kaolin, DNA adducts

Introduction

Recently, nanomaterials are being utilized for cosmetics and industrial products, and applications in medicine are under consideration. The assessment of genotoxicity and safety of nanomaterials is therefore of concern. One reason behind this is the asbestos crisis (1). Some nanomaterials are not only nano-sized particles, but also asbestos shape-like fibers, and the carcinogenic potential of such nanomaterials has attracted much attention over the years. Moreover, it is thought that nano-sized particles can be taken up in cells and cause intracellular damage (2,3). With this background, we here investigated induction of *in vitro* and *in vivo* genotoxicity using fullerene (C_{60}) and kaolin as examples. To clarify the mechanisms of mutations due to these nanomaterials, we analyzed the formation of DNA adducts in the lungs of mice after exposure. Here, we briefly summarize our data and also discuss mechanisms of genotoxicity induced by nanomaterials.

Size Distribution in Suspensions of Nanomaterials

The size distribution of nanomaterials used in the present study was analyzed by dynamic light scattering (DLS) as described previously (4). The most abundant sizes were at 234.1 ± 48.9 and 856.5 ± 119.2 nm for C_{60} and 357.6 ± 199.4 nm for kaolin, respectively.

In Vitro Genotoxicity Test

Micronucleus test: The micronucleus genotoxicity/clastogenicity test is widely used for assessment of environmental substances and medicinal chemicals. Here, we investigated the micronucleus inducing activity of C_{60} and kaolin using human lung carcinoma A549

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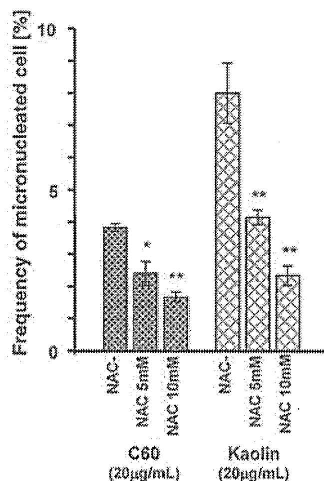

Fig. 1. Effects of anti-oxidative agents on the micronucleus inducing activity of nanoparticles. Values represent the means of three experiments ± SD. Asterisks (*, ** for $p<0.05$ and $p<0.01$, respectively) indicate significant differences from cells without NAC in the Student's t-test. Concentrations of nanoparticles in $\mu g/cm^2$ are given in parentheses.

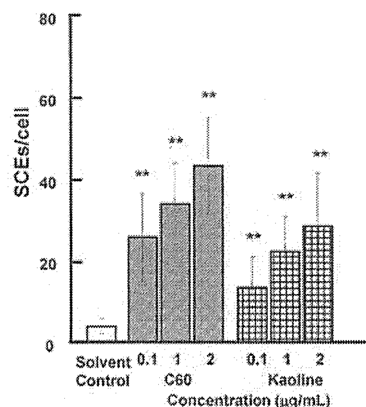

Fig. 2. Sister chromatid exchange (SCE) in CHO AA8 cells following treatment with C60 or kaolin for 1 h. The values represent the means of three experiments ± SD. Asterisks (**) indicate a significant difference ($p<0.01$) from control (treatment with 0.005% (v/v) Tween-80) cells in the Student's t-test.

cells (4). Six-hours treatment with 200 µg/mL kaolin caused growth inhibition of 60% whereas, C_{60} at the same concentration was without effect. C_{60} and kaolin particles both increased the number of micronucleated cells. The background frequency of micronucleated cells was 0.7% to 1.0%, and this rose to 10% and 5% with 200 µg/mL of C_{60} and kaolin, respectively, the increase being statistically significant in both cases. To investigate the effects of an anti-oxidative agent on the micronucleus induction, we conducted tests with or without *N*-acetyl cysteine (NAC) using Chinese hamster ovary CHO-AA8 cells. As shown in Fig. 1, the frequency of micronucleated cells was decreased significantly in the presence of NAC. With 20 µg/mL of C_{60} and kaolin for 6 h without NAC the results were 3.8% and 8%, respectively, but in the presence of 10 mM NAC these decreased to 1.7% and 2.3%. From this observation, oxidative stress might be involved in the genotoxicity induced by nanoparticles. Furthermore, it is known that photoexcited C_{60} produces reactive oxygen species (5) and in the present experiments, the cells and C_{60} were not shielded from visible light completely. Therefore, reactive oxygen species might contribute to micronucleus-induction in C_{60}-treated cells.

On the other hand, biologically relevant features of kaolin are unclear and further studies will be required to elucidate genotoxic mechanisms.

Sister chromatid exchange (SCE) test: SCE is also used for mutagenic testing of many products. While the mechanisms responsible for SCE are not completely understood, they involve breakage of both DNA strands, followed by exchange of whole DNA duplexes. This occurs during the S phase and is efficiently induced by mutagens that form DNA adducts or that interfere with DNA replication. To investigate SCE inducing activity of nanoparticles, we examined CHO-AA8 cells following 1 h treatment with C_{60} and kaolin (Fig. 2). The SCE frequencies in cells treated with 2.0 µg/mL of C_{60} and kaolin were approximately 11 and 7 times higher than the control level, respectively ($P<0.01$ at 0.1 µg/mL or higher concentrations). C_{60} demonstrated stronger genotoxic/clastogenic potency than kaolin. Cozzi *et al.* earlier reported that H_2O_2-treatment produced reactive oxygen species and induced SCE in CHO cells, and antioxidants, such as ascorbic acid and β-carotene, reduced the frequency (6). In the present study, the results of the micronucleus test indicated involvement of reactive oxygen species so that they might contribute to SCE induction as well.

In Vivo Genotoxicity Test

Comet assay: The comet assay is known as a standard simple and sensitive technique for evaluation of

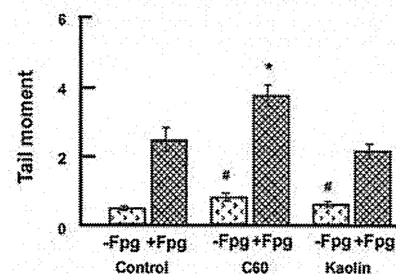

Fig. 3 DNA damage measured by comet assay in lungs of C57BL/6J mice intratracheally instilled with particles, with or without FPG treatment. Male mice were treated at a dose of 0.2 mg of particles per animal, and sacrificed 3 h after particle administration. The values represent the means of data for five animals ± SE. An asterisk (*) denotes $p<0.01$ from that of control (+FPG) and a sharp (#) denotes $p<0.01$ from that of control (-FPG) in a Dunnett's test after one-way ANOVA of Tail Moment.

DNA damage. The types of damage usually detected are single and double strand breaks. The pH (usually between neutral and alkaline pH) of the lysis condition can be adjusted depending upon the type of damage. Under alkaline conditions, AP sites and others where excision repair takes place are detected as DNA damage. We here evaluated DNA damage induced by particles using the comet assay under alkaline conditions. The values for DNA tail moment in the lungs with single-particle treatment at 0.2 mg/body for 3 h were measured, and DNA damage was significantly increased, around 2-fold, as compared with the vehicle control, and its intensity was C_{60} > kaolin. When we examined the effects of oxidation of purines, DNA damage was analyzed by formamidopyrimidin-glycosilase (FPG)-modified comet assay. DNA damage induced by kaolin was not changed, whereas DNA damage caused by C_{60} was elevated up to 1.7 fold compared with the vehicle control (Fig. 3). In addition, Jacobsen *et al.* also reported that C_{60} significantly increased the level of FPG sensitive sites/oxidized purines determined by the comet assay using the E1-Mutatrade markMouse lung epithelial cell line (7). From these findings, it seems that oxidative damage would be partly involved in the induction of DNA damage by C_{60}, although other changes responsible for DNA damage might be induced by kaolin.

Oxidative and lipid peroxide related DNA adduct formation: DNA adducts, formed by reactions with exogenous or endogenous agents, are known to induce gene mutations. Reactive oxygen species (ROS) are one type of endogenous agent that can produce oxidative DNA adducts such as 8-oxo-2′-deoxyguanosine (8-oxodG), a widely recognized and utilized biomarker of oxidative stress, and a major mutagenic lesion producing predominately G to T transversion mutations (8). In addition, ROS generate lipid hydroperoxides to yield heptanon-etheno (Hε)-adducts, such as HεdG, HεdA and HεdC via 4-oxo-2-nonenal (4-ONE) (9). These adducts can lead to mutations, if not repaired. We examined whether these oxidative and lipid peroxide related DNA adducts were induced in the lungs of mice by intratracheally instilled nanomaterials. 8-OxodG and three kinds of Hε-adducts were analyzed in the lungs of ICR mice 3, 24, 72 and 168 h after intratracheal instillation of 0.2 mg/body of C_{60} or kaolin, and quantified by the stable isotope dilution LC-MS/MS method described by Chou *et al.* (10). Compared with a vehicle control, DNA adduct levels were increased by about 2- to 5-fold in the lungs of mice 24 h after injection of nanoparticles (Fig. 4). The increases were time dependent until 72 h then gradually decreased within 168 h of injection (data not shown). Related to this, oxidative DNA damage was induced by intratracheal instillation of C_{60} or kaolin in the comet assay with FPG treatment, as described above. In addition, Folkmann *et al.* reported that oral gavage of C_{60} increased the levels of 8-oxodG in the liver and the lungs of F344 rats (11). Moreover, Tsurudome *et al.* described increased 8-oxodG levels induced by intratracheally instilled diesel exhaust particles in the lungs of F344 rats, and 8-oxoguanine DNA glycosylase 1 (OGG1) mRNA was also overexpressed (12). The decreased DNA adducts in the present study at 168 h may have been a result of a repair enzyme such as OGG1. This is the first observation that Hε-lipid peroxide related DNA adducts are increased by nanoparticles. Such adducts could clearly contribute to nanomaterial-induced DNA damage and mutation. Our findings suggest involvement of ROS generation, although differences between C_{60} and kaolin still require clarification.

***gpt* Mutations in the lungs of *gpt* transgenic mice:** Transgenic *gpt* delta mice are a useful model system for detecting both point mutations and large deletions (< 10 kb) (13). λEG10 transgenes carrying gpt (detection of point mutations) and *red*, *gam* (detection of deletion) genes have been integrated into mouse chromosome 17, and point mutations and deletions observed in any tissues can be detected as 6-thioguanine (6-TG) resistant colonies and Spi^- plaques, respectively. To examine *in vivo* mutagenicity of nanoparticles, *gpt* delta transgenic mice were exposed to C_{60} and kaolin at four different doses by intratracheal instillation, and *gpt* mutations were analyzed. The background *gpt* mutant frequency (MF) in lungs was $10.3 \pm 0.53 \times 10^{-6}$. MFs were significantly increased by 2 to 3-fold to $30.75 \pm 3.32 \times 10^{-6}$ (p=0.019) for C_{60} and $19.30 \pm 4.82 \times 10^{-6}$ (p=0.002) for kaolin (4).

Moreover, we examined the mutational characteris-

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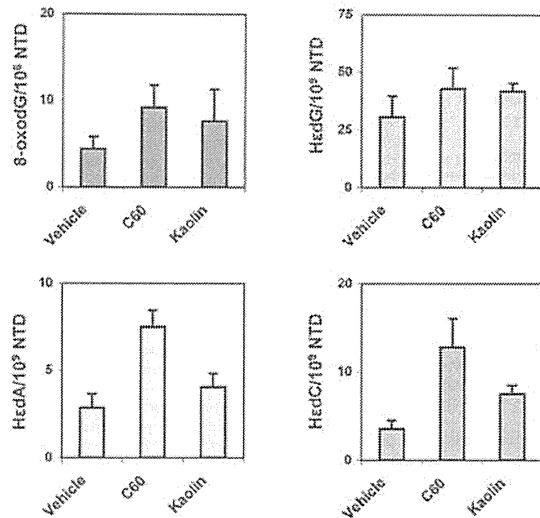

Fig. 4. Oxidative and lipid peroxide related DNA adduct formation in the lungs of ICR mice induced by nanoparticle exposure. DNA was extracted from lungs of mice 24 h after intratracheal instillation of 0.2 mg/body of C60 or kaolin, and digested enzymatically. Control animals were exposed to saline containing 0.05% Tween80. The 8-oxodG and 3 kinds of Hε-adducts were quantified by the stable isotope dilution LC-MS/MS method described by Chou *et al.* (10).

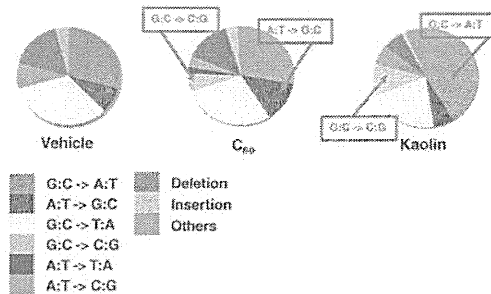

Fig. 5. Classification of *gpt* mutations from the lungs of control and nanoparticle treated mice.

tics induced by particles by PCR and DNA sequencing analysis of 6-TG resistant mutants. Classes of mutations found in the *gpt* gene are shown in Fig. 5. Interestingly, G:C to C:G transversions were increased in common with both particle treatments. Since these mutations were commonly increased regardless of the constituents (i.e., C_{60} is graphite and kaolin is aluminum silicate), the mechanisms might be the same. It has been reported that various oxidative stresses caused by sunlight, UV radiation, hydrogen peroxide and peroxy radicals frequently induce G:C to C:G transversions in various *in vitro* assay systems (14–17). Moreover, a variety of ox-

idative lesion products of guanine other than 8-oxodG, including imidazolone (Iz), oxazolone (Oz), spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh), have been reported (18–24). Three such molecules, Oz, Sp and Gh are now thought to be key causes G to C transversions with translesion synthesis systems (22–25). Therefore, it is suggested that G:C to C:G transversions induced by C_{60} and kaolin could involve Oz, Sp and Gh formation. In addition, G:C to A:T transitions were also significantly increased by instillation of kaolin but not C_{60}. In general, G to A (or C to T) transitions have commonly been observed in spontaneous and chemically-induced mutants, and deamination of guanine or 5-methylcytosine might be involved. Burney *et al.* reported that nitric oxide induces DNA damage. $NO^{\cdot}$ can form N_2O_3, and direct by this agent can lead to DNA deamination via diazonium ion formation (26). Moreover, nitric oxide is produced by activated macrophages in inflamed organs. In fact, test substance-phagocytized macrophages and granulomas were frequently observed in the lungs of mice (4).

Immunohistochemical Analysis of Inflammation Factors

In order to confirm enhancement of nitric oxide production by C_{60} and kaolin, we examined immunohistochemical staining of an inflammation factor, nitrotyrosine (NT), in the lungs of *gpt* delta mice treated with these nanoparticles using the same procedure reported previously (27) with minor modification. As shown in Fig. 6, the pattern of NT staining corresponded to the areas of inflammation within lung parenchyma. In the case of C_{60} exposure, many regions of the lungs stained positively (data not shown), and intense NT staining was localized in test substance-phagocytized macrophages and granulomas. Similarly, staining with NT antibodies was observed in macrophages and alveolar epithelial cells in the lungs of mice exposed to kaolin, although to a lesser extent as compared with C_{60}.

Conclusion

Our results clearly demonstrated that both *in vitro* and *in vivo* genotoxicity are induced by C_{60} and kaolin. However, the mechanisms have yet to be fully clarified, and oxidative stress might be at least partly involved. There are a number of ways in which reactive oxygen species (ROS) could be generated: i) nanoparticles might trigger ROS production by iron-catalysed Fenton reactions; ii) nanoparticles could accumulate in cells due to phagocytosis, then enhance the production of ROS by NADPH oxidase (28,29). Recently, innate immune activation through Nalp3 inflammasomes has been suggested to play an important role in pulmonary fibrotic disorders of silicosis and asbestosis (30,31). It has been reported that proinflammatory cytokines, such as interleukin 1β are key molecules for pneumoconiosis. At

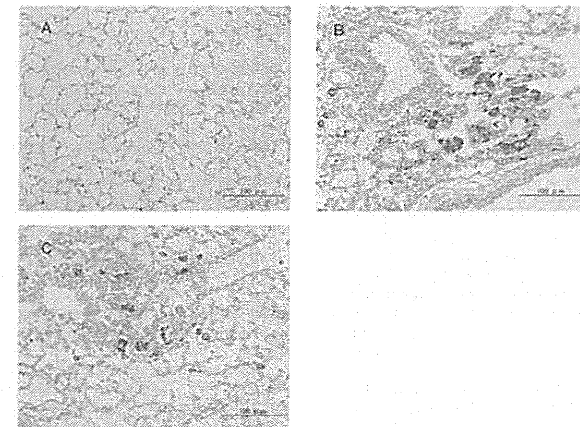

Fig. 6. Immunohistochemical localization of nitrotyrosine (NT). Since C_{60} is brown in color, we used an SG substrate kit (Vector Laboratories, USA) for peroxidase, with positive cells stained dark blue-gray. A: alveolar region in a control mouse, with no significant staining for NT. B: alveolar region in a mouse exposed to C_{60}, with positive macrophages phagocytizing test substance and epithelial cells. The brown colored material is C_{60}. C: alveolar region in a mouse exposed to kaolin. Note intense staining for NT in the granulomatous region.

present, no data are available for activation of the Nalp3 inflammasome pathway by C_{60} and kaolin. However, it is likely that both nanoparticles can activate in the same way as asbestos and silica, because oxidative stress was increased in the lungs of treated mice. Further studies of the mechanisms of genotoxicity are needed.

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Epidemiology Note

Cancer Incidence and Incidence Rates in Japan in 2006: Based on Data from 15 Population-based Cancer Registries in the Monitoring of Cancer Incidence in Japan (MCIJ) Project

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The Japan Cancer Surveillance Research Group estimated the cancer incidence in 2006 as part of the Monitoring of Cancer Incidence in Japan (MCIJ) project, on the basis of data collected from 15 of 32 population-based cancer registries. The total number of incidences in Japan for 2006 was estimated as 664 398 (C00–C96). The leading cancer site was stomach for men and breast for women. Age-standardized incidence rates remained at almost the same level as for the previous 3 years.

Key words: cancer incidence – incidence estimates – cancer registry – Japan

The Japan Cancer Surveillance Research Group is involved in cancer monitoring in Japan since 2000 (1–6). This group estimated the cancer incidence in 2006 as part of the Monitoring of Cancer Incidence in Japan (MCIJ) project, on the basis of data collected from 15 of 32 population-based cancer registries: Miyagi, Akita, Yamagata, Tochigi, Chiba, Kanagawa, Niigata, Fukui, Aichi, Tottori, Okayama, Hiroshima, Saga, Nagasaki and Kumamoto.

If data from all 32 registries were used, this would have led to a large underestimation of the national cancer incidence because of poor registration. The methods of registry selection, estimation of incidence and the limitations of these methods have been explained in previous studies (7–9). We maintained the same methodology since the MCIJ2003: (1) we invited all 32 population-based cancer registries in Japan to participate, and from these we selected the 15 cancer registries with high-quality data, which cover 33.7% of the total population in Japan; in order to estimate the national incidence, (2) we used 2006 data alone for the national estimation.

Through estimation, we consider those who meet the following standards as belonging to the 'high-quality' data area: DCO% (death certificate only: proportion of patients for whom the death certificate provides the only notification to the registry) <25% or DCN% (death certificate notification: proportion of patients for whom the death certificate provides the first notification to the registry) <30%, and IM ratio (incidence to mortality ratio) >1.5.

In estimating cancer incidence, in order to avoid a bias caused by the size of the registry population, an arithmetic mean of incidence rates of all the eligible registries (by primary site, sex and 5-year age group) was used, instead of dividing the total incidence by the total population. Cancer mortality in Japan was estimated by using the exact methods employed for the estimation of incidence, by taking mortality data from the vital statistics of the same eligible registries. Correction coefficients were then computed according to primary site and sex by using the observed cancer deaths divided by the number of estimated deaths. The estimated uncorrected cancer incidence

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according to primary site, sex and 5-year age group were multiplied by the correction coefficients to obtain corrected estimates. These corrected figures were then multiplied by the corresponding Japanese population to obtain the incidence figures for all Japan.

For this year, data from the Osaka prefecture, regularly considered as one of the registries with high quality, were not available for the MCIJ project. The other registries remained since the previous estimation in 2005, and Akita, Tochigi and Saga joined the registries for the national estimation.

The number of incidences, crude rates, age-standardized rates and completeness of registration in 2006 are shown in Table 1, and the age-specific number of incidences and the rates according to sex and primary site are shown in Tables 2 and 3. The total number of incidences in Japan for 2006 was estimated as 664 398 (C00–C96). The time trends of age-standardized incidence rates for the five major sites and male- and female-specific sites in 1975–2006 are shown in Fig. 1 (standard population: the world population) and in Fig. 2 (standard population: the 1985 Japanese model population). The leading cancer site according to the crude and age-standardized incidence rates was stomach followed by colon and rectum and lung for men and breast followed by colon and rectum and stomach for women since the research group took over national estimation of incidence, as shown in Table 1. Since the early 2000s, the age-standardized incidence rates in all cancer sites slightly increased, whereas they remained almost flat in the 1990s. The slight increase is thought to be due to the substantive increase of incidence in some primary sites, and partly due to improvement in the completeness of registry data in the 2000s. The estimated cancer incidence data in Japan by sex, site, 5-year age group and calendar year during the period 1975–2006 are available as a booklet, and as an electronic database on the website (only available in Japanese, http://ganjoho.jp/professional/statistics/monita.html).

Table 1. Incidence, completeness of reporting and accuracy of diagnosis in Japan according to sex and primary site, 2006

Primary sites	ICD-10th	Number of incidences	Crude rate[a]	Age-standardized rate[a]		Indices of data quality		
				World population	Japanese 1985 model population	DCO/I (%)	I/M	MV/I (%)
Male								
All sites (incl. CIS)	C00–C96, D00–D09	400 605	642.7	287.3	407.1	10.6	2.02	52.9
All sites	C00–C96	388 496	623.3	278.0	394.3	10.7	1.96	52.6
Lip, oral cavity and pharynx	C00–C14	9130	14.6	7.3	9.9	9.4	2.12	81.0
Esophagus	C15	15 818	25.4	11.7	16.2	10.9	1.64	80.8
Stomach	C16	79 437	127.4	56.9	80.8	11.3	2.43	81.9
Colon and rectum	C18–C20	62 116	99.7	45.7	64.1	9.8	2.77	84.5
Colon	C18	38 182	61.3	27.3	38.8	10.4	2.79	81.4
Rectum	C19	23 934	38.4	18.4	25.4	9.1	2.75	83.1
Liver	C22	28 872	46.3	20.9	29.5	16.6	1.28	44.3
Gallbladder, etc.	C23–C24	9740	15.6	6.1	9.2	20.7	1.23	43.4
Pancreas	C25	13 768	22.1	9.6	13.8	21.8	1.10	41.5
Larynx	C32	3447	5.5	2.5	3.5	9.5	3.66	74.7
Trachea, bronchus and lung	C33–C34	59 934	96.2	39.2	58.2	14.6	1.30	74.2
Melanoma of skin, etc.	C43–C44	4660	7.5	3.2	4.6	7.7	7.56	88.6
Prostate	C61	42 517	68.2	27.1	40.2	9.1	4.46	86.0
Bladder	C67	12 478	20.0	8.3	12.2	9.7	2.96	82.0
Kidney, renal pelvis, ureter, etc.	C64–C66, C68	9608	15.4	7.4	10.2	10.1	2.40	76.1
Brain and nervous system	C70–C72	2491	4.0	2.8	3.2	14.4	2.60	71.8
Thyroid	C73	2382	3.8	2.3	2.9	4.8	4.78	86.0
Malignant lymphoma	C81–C85, C96	9867	15.8	8.0	10.8	9.3	1.97	88.8
Multiple myeloma	C88, C90	2505	4.0	1.6	2.4	16.3	1.32	75.2
All leukaemias	C91–C95	5544	8.9	5.2	6.5	16.5	1.27	84.4

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Table 1. *Continued*

Primary sites	ICD-10th	Number of incidences	Crude rate[a]	Age-standardized rate[a]		Indices of data quality		
				World population	Japanese 1985 model population	DCO/I (%)	I/M	MV/I (%)
Female								
All sites (incl. CIS)	C00–C96, D00–D09	293 179	448.0	205.8	274.6	12.4	2.23	76.9
All site	C00–C96	275 902	421.6	187.3	251.8	13.2	2.10	75.6
Lip, oral cavity and pharynx	C00–C14	3496	5.3	2.2	3.1	12.4	2.05	78.7
Esophagus	C15	2905	4.4	1.6	2.3	14.1	1.71	78.9
Stomach	C16	37 474	57.3	21.2	29.7	15.9	2.12	78.4
Colon and rectum	C18–C20	44 788	68.4	25.9	36.1	12.5	2.40	81.9
Colon	C18	31 719	48.5	17.6	24.7	13.5	2.33	77.6
Rectum	C19	13 069	20.0	8.3	11.3	11.4	2.60	80.4
Liver	C22	14 021	21.4	6.8	10.0	21.7	1.26	40.1
Gallbladder, etc.	C23–C24	10 358	15.8	4.3	6.5	26.7	1.16	35.6
Pancreas	C25	11 722	17.9	5.6	8.2	27.3	1.08	33.0
Larynx	C32	278	0.4	0.2	0.2	11.6	4.56	73.3
Trachea, bronchus and lung	C33–C34	25 543	39.0	13.9	19.6	21.2	1.48	60.3
Melanoma of skin, etc.	C43–C44	3930	6.0	2.1	2.8	9.7	6.09	86.8
Breast (incl. CIS)	C50, D05	53 783	82.2	51.0	65.6	4.3	4.81	91.0
Breast (only invasive)	C50	49 772	76.1	46.8	60.3	4.6	4.45	90.2
Uterus (incl. CIS)	C53–C55, D06	25 859	39.5	28.0	34.7	4.9	4.69	89.9
Uterus (only invasive)	C53–C55	18 642	28.5	17.9	22.8	6.1	3.38	87.4
Cervix uteri	C53	8968	13.7	9.5	12.0	5.3	3.61	88.4
Corpus uteri	C54	8629	13.2	7.9	10.1	3.7	5.83	90.9
Ovary	C56	7913	12.1	7.2	9.1	10.3	1.78	80.0
Bladder	C67	4032	6.2	1.8	2.7	14.0	2.11	75.3
Kidney, renal pelvis, ureter, etc.	C64–C66, C68	5278	8.1	3.0	4.3	14.8	2.30	70.7
Brain and nervous system	C70–C72	2217	3.4	1.9	2.3	16.3	3.04	65.2
Thyroid	C73	7852	12.0	7.4	9.4	7.1	7.53	85.9
Malignant lymphoma	C81–C85, C96	8769	13.4	6.1	8.0	10.2	2.37	87.1
Multiple myeloma	C88, C90	2304	3.5	1.2	1.7	20.1	1.18	68.6
All leukaemias	C91–C95	3835	5.9	3.4	3.9	19.6	1.26	83.5

ICD-10th, *International Classification of Diseases*, 10th Revision; DCO/I, proportion of cases with the death certificate only to incident cases; I/M, number of incidences/number of deaths; MV/I, proportion of microscopically verified cases to incident cases; CIS, carcinoma *in situ*.
[a]Per 100 000 population.

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Conflict of interest statement

None declared.

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Table 2. Age-specific incidence in Japan according to sex and primary site, 2006

Primary sites	ICD-10th	Age group (years)																	
		0–4	5–9	10–14	15–19	20–24	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–79	80–84	85+
Male																			
All sites (incl. CIS)	C00–C96, D00–D09	310	178	232	250	682	831	1646	2780	4530	8088	15 994	37 893	45 699	60 319	74 748	70 466	44 689	31 270
All sites	C00–C96	310	178	232	250	682	819	1594	2703	4326	7762	15 304	36 250	44 007	58 233	72 418	68 673	43 861	30 894
Lip, oral cavity and pharynx	C00–C14	0	0	7	6	18	54	104	127	137	297	675	1307	1320	1512	1435	1175	599	357
Esophagus	C15	0	0	0	1	0	4	0	13	104	283	749	2042	2498	2929	2893	2304	1221	777
Stomach	C16	0	0	0	2	31	61	192	490	823	1818	3462	8381	9209	12 708	14 940	13 466	8247	5607
Colon and rectum	C18–C20	1	0	14	10	22	80	207	553	861	1600	3014	6717	7618	10 000	11 212	10 086	5784	4337
Colon	C18	1	0	14	10	6	59	101	357	469	775	1517	3546	4460	6051	7168	6711	3851	3086
Rectum	C19	0	0	0	0	16	21	106	196	392	825	1497	3171	3158	3949	4044	3375	1933	1251
Liver	C22	24	4	0	0	5	22	49	112	329	577	1327	3010	3647	4493	6286	4799	2602	1586
Gallbladder, etc.	C23–C24	0	0	0	0	0	16	2	37	27	70	225	541	772	1171	1704	2058	1554	1563
Pancreas	C25	0	0	1	0	0	1	13	42	105	324	602	1284	1681	2000	2215	2423	1693	1384
Larynx	C32	0	0	0	0	0	0	3	18	12	52	177	329	570	683	629	559	265	150
Trachea, bronchus and lung	C33–C34	5	0	0	1	30	26	59	179	365	809	1627	4490	5933	7781	11 021	12 384	9161	6063
Melanoma of skin, etc.	C43–C44	6	1	1	10	15	18	62	53	43	78	186	359	373	473	744	853	709	676
Prostate	C61	0	0	0	0	1	0	12	19	16	74	480	2079	4249	7079	10 252	9286	5337	3633
Bladder	C67	1	0	0	0	23	10	8	25	103	253	368	1057	1211	1490	2089	2474	1917	1449
Kidney, renal pelvis, ureter, etc.	C64–C66, C68	14	10	12	0	0	24	39	121	191	304	539	1165	1247	1364	1671	1396	956	555
Brain and nervous system	C70–C72	47	70	66	39	64	35	51	152	105	107	149	182	254	352	342	280	141	55
Thyroid	C73	0	0	0	5	32	55	103	121	140	129	163	299	334	304	312	204	84	97
Malignant lymphoma	C81–C85, C96	11	10	29	37	78	121	142	188	278	343	549	1133	992	1319	1456	1451	1079	651
Multiple myeloma	C88, C90	0	0	0	0	0	0	5	2	35	37	45	189	228	354	461	509	398	242
All leukaemias	C91–C95	89	60	50	65	114	79	126	131	218	172	269	498	501	660	779	830	545	358

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Female																			
All sites (incl. CIS)	C00–C96, D00–D09	215	167	177	346	1107	2706	5038	7522	10 598	14 515	18 482	28 369	26 894	30 850	37 506	36 649	31 881	40 157
All site	C00–C96	215	167	177	332	763	1659	3427	5650	9066	13 024	17 301	27 011	25 515	29 221	36 123	35 424	31 101	39 726
Lip, oral cavity and pharynx	C00–C14	0	0	2	12	5	24	42	90	111	174	161	308	231	303	528	471	452	582
Esophagus	C15	0	0	0	0	0	0	0	26	24	68	98	354	331	255	410	431	342	566
Stomach	C16	0	0	0	10	36	90	212	407	626	971	1572	3003	3038	4113	5553	5576	5422	6845
Colon and rectum	C18–C20	0	8	0	8	26	71	165	327	628	1158	2030	3825	4287	5490	6679	6834	5830	7422
Colon	C18	0	8	0	8	18	53	114	179	396	667	1272	2597	2834	3749	4766	4999	4261	5798
Rectum	C19	0	0	0	0	8	18	51	148	232	491	758	1228	1453	1741	1913	1835	1569	1624
Liver	C22	4	0	0	0	8	10	40	42	37	109	217	576	1044	1694	2822	2917	2241	2260
Gallbladder, etc.	C23–C24	0	0	0	0	0	4	2	18	5	98	151	384	554	888	1475	1705	2044	3030
Pancreas	C25	0	0	1	4	6	0	18	27	39	153	339	633	886	1211	1782	2021	1988	2614
Larynx	C32	0	0	0	0	0	0	0	0	1	0	13	26	58	25	42	32	40	41
Trachea, bronchus and lung	C33–C34	0	0	0	1	15	29	76	138	252	467	969	2162	2248	2915	4098	3974	3341	4858
Melanoma of skin, etc.	C43–C44	0	9	0	9	33	42	34	49	24	74	142	240	188	388	406	465	605	1222
Breast (incl. CIS)	C50, D05	0	0	0	3	12	277	987	2371	4652	6629	6479	7865	6576	5754	4685	3511	2159	1823
Breast (only invasive)	C50	0	0	0	3	12	243	932	2148	4245	5930	5880	7414	6112	5322	4405	3258	2065	1803
Uterus (incl. CIS)	C53–C55, D06	0	0	0	15	466	1448	2455	2841	2409	2075	2584	3140	2066	1720	1518	1195	921	1006
Uterus (only invasive)	C53–C55	0	0	0	1	160	444	944	1293	1450	1486	2306	2807	1830	1515	1400	1109	895	1002
Cervix uteri	C53	0	0	0	1	127	411	814	1039	1018	828	712	1010	554	531	616	430	443	434
Corpus uteri	C54	0	0	0	0	33	33	128	242	426	637	1540	1748	1221	901	696	529	265	230
Ovary	C56	1	1	36	56	97	93	166	241	433	672	992	1161	895	725	750	585	417	592
Bladder	C67	0	0	0	1	1	17	14	6	9	30	79	225	228	388	666	615	765	988
Kidney, renal pelvis, ureter, etc.	C64–C66, C68	9	0	0	0	0	9	26	49	89	135	225	444	357	595	960	895	680	805
Brain and nervous system	C70–C72	26	41	32	27	38	66	62	34	62	86	106	238	124	179	291	379	212	214
Thyroid	C73	0	0	0	41	103	210	374	412	518	627	750	1134	847	798	754	591	372	321
Malignant lymphoma	C81–C85, C96	37	25	2	20	71	111	78	131	172	318	533	835	967	867	1203	1121	1159	1119
Multiple myeloma	C88, C90	0	0	0	0	0	0	0	0	20	37	87	118	241	252	332	432	375	410
All leukaemias	C91–C95	92	66	23	71	71	86	70	77	113	103	152	347	265	436	410	591	390	472

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Table 3. Age-specific incidence rate per 100 000 population in Japan according to sex and primary site, 2006

Primary sites	ICD-10th	Age group (years)																	
		0–4	5–9	10–14	15–19	20–24	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–79	80–84	85+
Male																			
All sites (incl. CIS)	C00–C96, D00–D09	11.0	5.9	7.5	7.6	18.2	20.4	33.7	59.4	112.7	209.6	380.9	706.8	1153.4	1655.3	2386.6	3019.1	3310.3	3678.8
All sites	C00–C96	11.0	5.9	7.5	7.6	18.2	20.1	32.6	57.7	107.6	201.2	364.5	676.2	1110.7	1598.1	2312.2	2942.3	3249.0	3634.6
Lip, oral cavity and pharynx	C00–C14	0.0	0.0	0.2	0.2	0.5	1.3	2.1	2.7	3.4	7.7	16.1	24.4	33.3	41.5	45.8	50.3	44.4	42.0
Esophagus	C15	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.3	2.6	7.3	17.8	38.1	63.0	80.4	92.4	98.7	90.4	91.4
Stomach	C16	0.0	0.0	0.0	0.1	0.8	1.5	3.9	10.5	20.5	47.1	82.4	156.3	232.4	348.7	477.0	576.9	610.9	659.6
Colon and rectum	C18–C20	0.0	0.0	0.5	0.3	0.6	2.0	4.2	11.8	21.4	41.5	71.8	125.3	192.3	274.4	358.0	432.1	428.4	510.2
Colon	C18	0.0	0.0	0.5	0.3	0.2	1.4	2.1	7.6	11.7	20.1	36.1	66.1	112.6	166.1	228.9	287.5	285.3	363.1
Rectum	C19	0.0	0.0	0.0	0.0	0.4	0.5	2.2	4.2	9.8	21.4	35.7	59.1	79.7	108.4	129.1	144.6	143.2	147.2
Liver	C22	0.9	0.1	0.0	0.0	0.1	0.5	1.0	2.4	8.2	15.0	31.6	56.1	92.0	123.3	200.7	205.6	192.7	186.6
Gallbladder, etc.	C23–C24	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.8	0.7	1.8	5.4	10.1	19.5	32.1	54.4	88.2	115.1	183.9
Pancreas	C25	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.9	2.6	8.4	14.3	24.0	42.4	54.9	70.7	103.8	125.4	162.8
Larynx	C32	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.4	0.3	1.3	4.2	6.1	14.4	18.7	20.1	24.0	19.6	17.6
Trachea, bronchus and lung	C33–C34	0.2	0.0	0.0	0.0	0.8	0.6	1.2	3.8	9.1	21.0	38.7	83.8	149.7	213.5	351.9	530.6	678.6	713.3
Melanoma of skin, etc.	C43–C44	0.2	0.0	0.0	0.3	0.4	0.4	1.3	1.1	1.1	2.0	4.4	6.7	9.4	13.0	23.8	36.5	52.5	79.5
Prostate	C61	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.4	0.4	1.9	11.4	38.8	107.2	194.3	327.3	397.9	395.3	427.4
Bladder	C67	0.0	0.0	0.0	0.0	0.6	0.2	0.2	0.5	2.6	6.6	8.8	19.7	30.6	40.9	66.7	106.0	142.0	170.5
Kidney, renal pelvis, ureter, etc.	C64–C66, C68	0.5	0.3	0.4	0.0	0.0	0.6	0.8	2.6	4.8	7.9	12.8	21.7	31.5	37.4	53.4	59.8	70.8	65.3
Brain and nervous system	C70–C72	1.7	2.3	2.1	1.2	1.7	0.9	1.0	3.2	2.6	2.8	3.5	3.4	6.4	9.7	10.9	12.0	10.4	6.5
Thyroid	C73	0.0	0.0	0.0	0.2	0.9	1.4	2.1	2.6	3.5	3.3	3.9	5.6	8.4	8.3	10.0	8.7	6.2	11.4
Malignant lymphoma	C81–C85, C96	0.4	0.3	0.9	1.1	2.1	3.0	2.9	4.0	6.9	8.9	13.1	21.1	25.0	36.2	46.5	62.2	79.9	76.6
Multiple myeloma	C88, C90	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.9	1.0	1.1	3.5	5.8	9.7	14.7	21.8	29.5	28.5
All leukaemias	C91–C95	3.2	2.0	1.6	2.0	3.0	1.9	2.6	2.8	5.4	4.5	6.4	9.3	12.6	18.1	24.9	35.6	40.4	42.1

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Female																			
All sites (incl. CIS)	C00–C96, D00–D09	8.0	5.8	6.0	11.1	31.1	68.7	105.9	163.8	267.4	378.3	438.0	519.2	643.2	775.1	1018.6	1190.3	1381.3	1788.7
All site	C00–C96	8.0	5.8	6.0	10.6	21.4	42.1	72.0	123.0	228.8	339.4	410.0	494.3	610.3	734.2	981.1	1150.5	1347.5	1769.5
Lip, oral cavity and pharynx	C00–C14	0.0	0.0	0.1	0.4	0.1	0.6	0.9	2.0	2.8	4.5	3.8	5.6	5.5	7.6	14.3	15.3	19.6	25.9
Esophagus	C15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.6	1.8	2.3	6.5	7.9	6.4	11.1	14.0	14.8	25.2
Stomach	C16	0.0	0.0	0.0	0.3	1.0	2.3	4.5	8.9	15.8	25.3	37.3	55.0	72.7	103.3	150.8	181.1	234.9	304.9
Colon and rectum	C18–C20	0.0	0.3	0.0	0.3	0.7	1.8	3.5	7.1	15.8	30.2	48.1	70.0	102.5	137.9	181.4	222.0	252.6	330.6
Colon	C18	0.0	0.3	0.0	0.3	0.5	1.3	2.4	3.9	10.0	17.4	30.1	47.5	67.8	94.2	129.4	162.4	184.6	258.3
Rectum	C19	0.0	0.0	0.0	0.0	0.2	0.5	1.1	3.2	5.9	12.8	18.0	22.5	34.8	43.7	52.0	59.6	68.0	72.3
Liver	C22	0.1	0.0	0.0	0.0	0.2	0.3	0.8	0.9	0.9	2.8	5.1	10.5	25.0	42.6	76.6	94.7	97.1	100.7
Gallbladder, etc.	C23–C24	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.4	0.1	2.6	3.6	7.0	13.3	22.3	40.1	55.4	88.6	135.0
Pancreas	C25	0.0	0.0	0.0	0.1	0.2	0.0	0.4	0.6	1.0	4.0	8.0	11.6	21.2	30.4	48.4	65.6	86.1	116.4
Larynx	C32	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.5	1.4	0.6	1.1	1.0	1.7	1.8
Trachea, bronchus and lung	C33–C34	0.0	0.0	0.0	0.0	0.4	0.7	1.6	3.0	6.4	12.2	23.0	39.6	53.8	73.2	111.3	129.1	144.8	216.4
Melanoma of skin, etc.	C43–C44	0.0	0.3	0.0	0.3	0.9	1.1	0.7	1.1	0.6	1.9	3.4	4.4	4.5	9.7	11.0	15.1	26.2	54.4
Breast (incl. CIS)	C50, D05	0.0	0.0	0.0	0.1	0.3	7.0	20.7	51.6	117.4	172.8	153.5	143.9	157.3	144.6	127.2	114.0	93.5	81.2
Breast (only invasive)	C50	0.0	0.0	0.0	0.1	0.3	6.2	19.6	46.8	107.1	154.5	139.3	135.7	146.2	133.7	119.6	105.8	89.5	80.3
Uterus (incl. CIS)	C53–C55, D06	0.0	0.0	0.0	0.5	13.1	36.7	51.6	61.9	60.8	54.1	61.2	57.5	49.4	43.2	41.2	38.8	39.9	44.8
Uterus (only invasive)	C53–C55	0.0	0.0	0.0	0.0	4.5	11.3	19.8	28.2	36.6	38.7	54.6	51.4	43.8	38.1	38.0	36.0	38.8	44.6
Cervix uteri	C53	0.0	0.0	0.0	0.0	3.6	10.4	17.1	22.6	25.7	21.6	16.9	18.5	13.3	13.3	16.7	14.0	19.2	19.3
Corpus uteri	C54	0.0	0.0	0.0	0.0	0.9	0.8	2.7	5.3	10.7	16.6	36.5	32.0	29.2	22.6	18.9	17.2	11.5	10.2
Ovary	C56	0.0	0.0	1.2	1.8	2.7	2.4	3.5	5.2	10.9	17.5	23.5	21.2	21.4	18.2	20.4	19.0	18.1	26.4
Bladder	C67	0.0	0.0	0.0	0.0	0.0	0.4	0.3	0.1	0.2	0.8	1.9	4.1	5.5	9.7	18.1	20.0	33.1	44.0
Kidney, renal pelvis, ureter, etc.	C64–C66, C68	0.3	0.0	0.0	0.0	0.0	0.2	0.5	1.1	2.2	3.5	5.3	8.1	8.5	14.9	26.1	29.1	29.5	35.9
Brain and nervous system	C70–C72	1.0	1.4	1.1	0.9	1.1	1.7	1.3	0.7	1.6	2.2	2.5	4.4	3.0	4.5	7.9	12.3	9.2	9.5
Thyroid	C73	0.0	0.0	0.0	1.3	2.9	5.3	7.9	9.0	13.1	16.3	17.8	20.8	20.3	20.1	20.5	19.2	16.1	14.3
Malignant lymphoma	C81–C85, C96	1.4	0.9	0.1	0.6	2.0	2.8	1.6	2.9	4.3	8.3	12.6	15.3	23.1	21.8	32.7	36.4	50.2	49.8
Multiple myeloma	C88, C90	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0	2.1	2.2	5.8	6.3	9.0	14.0	16.2	18.3
All leukaemias	C91–C95	3.4	2.3	0.8	2.3	2.0	2.2	1.5	1.7	2.9	2.7	3.6	6.4	6.3	11.0	11.1	19.2	16.9	21.0

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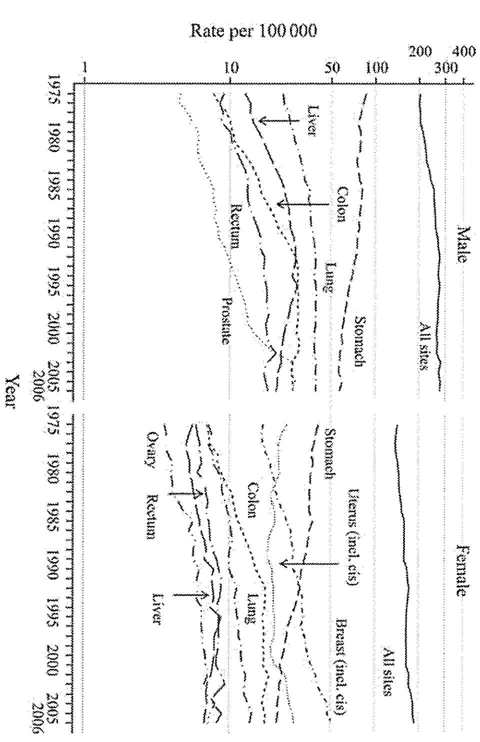

Figure 1. Trends of age-standardized cancer incidence rates for five major sites and specific sites for each sex (standard population: world population).

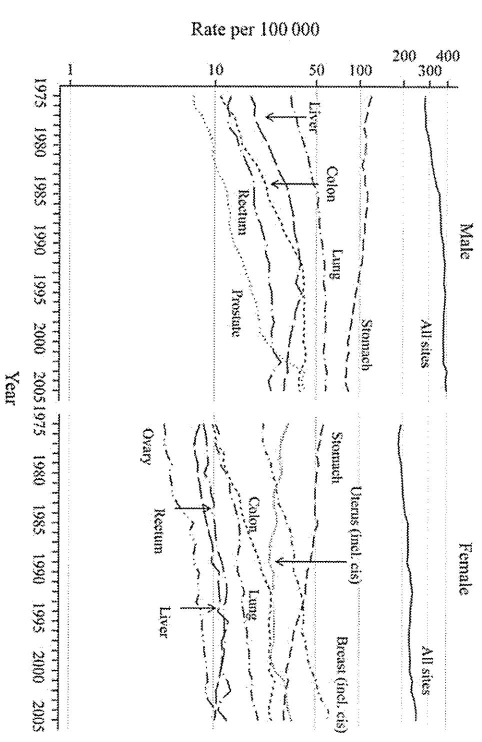

Figure 2. Trends of age-standardized cancer incidence rates for five major sites and specific sites for each sex (standard population: 1985 Japanese model population).

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Induction of glandular stomach cancers in *Helicobacter pylori*-infected Mongolian Gerbils by 1-nitrosoindole-3-acetonitrile

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Helicobacter pylori (*H. pylori*) infection and high intake of various traditional salt-preserved foods are regarded as risk factors for human gastric cancer. We previously reported that Chinese cabbage contains indole compounds, such as indole-3-acetonitrile, a mutagen precursor. 1-Nitrosoindole-3-acetonitrile (NIAN), formed by the treatment of indole-3-acetonitrile with nitrite under acidic conditions, shows direct-acting mutagenicity. In the present study, NIAN administration by gavage to Mongolian gerbils (MGs) at the dose of 100 mg/kg two times a week resulted in three adduct spots (1.6 adducts/10^8 nucleotides in total), detected in DNA samples from the glandular stomach by ^{32}P-postlabeling methods. Treatment with six consecutive doses of 100 mg/kg of NIAN, two times a week for 3 weeks, induced well—and moderately—differentiated glandular stomach adenocarcinomas in the MGs at the incidence of 31% under *H. pylori* infection at 54–104 weeks. Such lesions were not induced in MGs given broth alone, broth + NIAN or infection with *H. pylori* alone. Thus, endogenous carcinogens formed from nitrosation of indole compounds could be critical risk factors for human gastric cancer development under the influence of *H. pylori* infection.

Carcinogenesis

Gastric cancer is the second most frequent cause of cancer death worldwide.[1] Although gastric cancer has become a relatively rare cancer in North America and most Northern and Western European countries, it remains common in East Asia, Eastern Europe, Russia, and selected areas of Central and South America.[2] *Helicobacter pylori* (*H. pylori*) is a well-established major risk factor for gastric cancer,[3–5] and the prevalence of *H. pylori* infection in East Asia countries, including Japan and Korea is reported to be relatively high.[6,7] In addition, the risk of gastric cancer is increased with a high intake of various traditional salt-preserved foods.[3] In fact, pickled vegetable consumption is reported to increase gastric cancer risk in Japan and Korea.[8–10] In Korea, kimchi, commonly prepared with Chinese cabbage or radish, is a traditional and popular food, which contains high levels of nitrate (median 1550 mg/kg).[11] Furthermore, Chinese cabbage is well known as a pickled vegetable commonly consumed in Japan. Moreover, ingestion of nitrate, mainly from food, is suggested to correlate with mortality from gastric cancer.[12–14] Ingested nitrate is mainly converted to nitrite by bacteria in the oral cavity after secretion into saliva.[15] Carcinogenic *N*-nitroso compounds can be formed from nitrite and secondary amines under acidic conditions. Furthermore, direct-acting *N*-nitroso compounds, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)[16] and *N*-methyl-*N*-nitrosourea (MNU),[17] are known to induce cancer in the glandular stomach of experimental animals. Thus, it is suggested that *N*-nitroso compounds that are formed in the stomach under acidic conditions could be positively associated with the risk of gastric cancer. Nitric oxide, formed by nitric oxide synthase, is also reported to contribute to production of *N*-nitroso compounds.[18]

We have previously reported that treatments of various foodstuffs with nitrite under acidic conditions produce direct-acting mutagens towards *Salmonella* tester strains.[19,20] Among those foodstuffs, Chinese cabbage is shown to contain three indole compounds, indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde as mutagen precursors. 1-Nitrosoindole-3-acetonitrile (NIAN), an *N*-nitroso-substituted compound formed by treatment of indole-3-

Key words: gastric cancer, *Helicobacter pylori*, Mongolian gerbil 1-nitrosoindole-3-acetonitrile, indole-3-acetonitrile
Abbreviations: DMSO: dimethyl sulfoxide; H&E: hematoxylin and eosin; *H. pylori*: *Helicobacter pylori*; MG: Mongolian gerbil; MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU: *N*-methyl-*N*-nitrosourea; NIAN: 1-nitrosoindole-3-acetonitrile.
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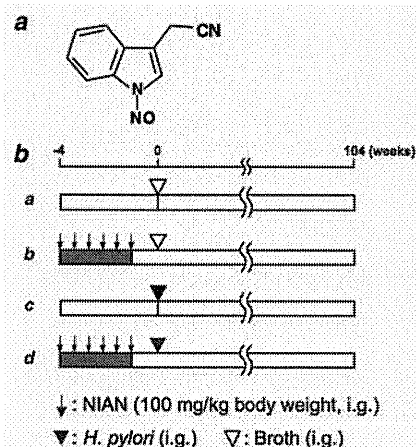

Figure 1. Chemical structure of NIAN and experimental protocol for the carcinogenicity study. (*a*) Chemical structure of NIAN. (*b*) Male 6-week-old MGs were orally administered NIAN (100 mg/kg) in 50% DMSO (groups B and D) or 50% DMSO alone (groups A and C) two times a week for 3 weeks. One week after the final administration, the animals were inoculated with *H. pylori* (ATCC 43504) (groups C and D) or sterilized broth (groups A and B).

acetonitrile with nitrite under acidic conditions, is a direct-acting mutagen in *S. typhimurium* and Chinese hamster lung cells,[20–22] and it is confirmed to form DNA adducts and to induce DNA single-strand scission in the rat glandular stomach.[23,24] Therefore, NIAN could play some role in gastric cancer development, as in the case of the well-known direct-acting mutagens, MNNG and MNU, in animal experiments.[16,17,25]

The Mongolian gerbil (MG) is reported to be susceptible to colonization by *H. pylori*, and *H. pylori* infection greatly enhances MNNG or MNU-induced gastric carcinogenesis in MGs.[26,27] Therefore, the MG is considered to be a useful animal model for evaluating the gastric cancer risk of direct-acting *N*-nitroso compounds, with or without *H. pylori* infection.

Chinese cabbage, containing nitrate and indole compounds, is commonly consumed in East Asian countries, including Japan, Korea and China, in which gastric cancer mortality is very high. In the present study, DNA adducts were detected with NIAN treatment in the glandular stomach of MGs, and the carcinogenicity of NIAN for gastric cancer *in vivo* was examined. The results clearly demonstrated that gastric cancer developed with a combination of NIAN administration and *H. pylori* infection in MGs. Possible involvement of indole compounds and nitrate derived from various foodstuffs, including Chinese cabbage, in gastric cancer development in humans is discussed.

Material and Methods

Materials

Indole-3-acetonitrile was purchased from Tokyo Food Techno (Tokyo, Japan), sodium nitrite from Wako Pure Chemical Industries (Osaka, Japan) and ammonium sulfamate from Kanto Chemical (Tokyo, Japan). Brucella broth was obtained from Becton Dickinson (Cockeysville, MD) and horse serum from Nippon Bio-Supply (Tokyo, Japan).

Preparation of NIAN

The chemical structure of NIAN is shown in Figure 1*a*. Indole-3-acetonitrile in 27 mM citrate-phosphate buffer (pH 3.0) was treated with 50 mM sodium nitrite for 1 hr at room temperature in the dark, as previously reported.[21] Nitrosation was stopped by addition of ammonium sulfamate at a final concentration of 50 mM. The reaction solution was filtered and the residue was washed with deionized water, then with *n*-hexane. The residual paste was dried and stored at −80°C until use. The preparation was >93% pure as judged by its UV absorbance on HPLC.

Bacterial culture

H. pylori (ATCC 43504; American Type Culture Collection, Manassas, VA) was cultured in brucella broth supplemented with 10% heat-inactivated horse serum for 24 hr at 37°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2), as previously described.[28]

Animal treatment

Specific pathogen-free male, 6-week-old MGs (MGS/Sea, Kyudo, Fukuoka, Japan) were housed in a biohazard room, air-conditioned at 24°C ± 2°C and 55% humidity, on a 12 hr light–dark cycle and were allowed free access to commercial diet (CE-2; CLEA Japan, Tokyo, Japan) and water.

To analyze the formation of DNA adducts in the glandular stomach of MGs by NIAN treatment, NIAN was dissolved in 50% dimethyl sulfoxide (DMSO), and administered to three MGs by gavage of 0.5 ml solution, two times a week at a level of 100 mg/kg body weight. Two further MGs served as a control group receiving the solvent alone (0.5 ml). At 8 hr after administration of NIAN, both groups of animals were sacrificed under ether anesthesia, and their stomachs were resected and stored at −80°C until use. DNA was extracted by a standard procedure with enzymatic digestion of protein and RNA followed by extraction with phenol and chloroform/isoamyl alcohol (24:1, v/v).

The protocol for long-term gastric carcinogenicity in MGs treated with NIAN + *H. pylori* infection is illustrated in Figure 1*b*. The animals were randomly divided into four groups (groups A–D). Groups A and C were given 50% DMSO without NIAN (0.5 ml) whereas groups B and D were orally administrated NIAN (0.5 ml, 100 mg/kg body weight) dissolved in 50% DMSO by gavage, two times a week for 3 weeks. At one week after the last administration, the

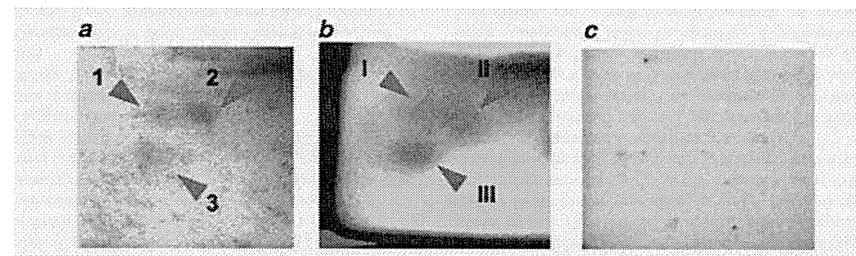

Figure 2. Autoradiograms of NIAN-DNA adducts in glandular stomach of MGs or calf thymus DNA treated with NIAN. Adducts were analyzed by ^{32}P-postlabeling method, as described in the Material and Methods. DNA samples were isolated from glandular stomach of MGs (*a*) or calf thymus DNA (*b*) after treatment with NIAN. DNA samples were also prepared from glandular stomach of MGs without NIAN treatment (*c*). Arrowheads indicate adducts. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

animals of groups C and D were given an intragastric inoculation of *H. pylori* broth culture (0.5 ml, 0.9 × 10^8 CFU/animal) whereas animals of groups A and B were given sterilized broth alone (0.5 ml).[28]

During the experiments, animals which became moribund or emaciated (<80 g body weight) were sacrificed. At 104 weeks after *H. pylori* infection, all surviving animals were sacrificed under ether anesthesia. At performance of necropsy, all tissues were carefully checked macroscopically and the stomachs and major organs were removed and assessed for macroscopic lesion development. Effective numbers of animals were defined as those surviving until week 54 of the study, when gastric tumors were observed for the first time. In addition, in the *H. pylori*-infected groups, the animals developing gastritis observed on histological examination were regarded as effective. The percentages of gastritis-bearing animals by the single inoculation of *H. pylori* were 62% for group C and 76% for group D, being similar to those previously reported.[27] All animal experiments were performed according to the "Guidelines for Animal Experiments in the National Cancer Center" and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

Detection of DNA adducts by ^{32}P-postlabeling method

Calf thymus DNA (0.5 mg, Sigma, St. Louis, MO) treated with NIAN (3 mg) for 12 hr under neutral conditions was used for authentic NIAN-DNA adducts.[23] DNA samples from the glandular stomach of MGs and calf thymus DNA samples were digested with micrococcal nuclease and phosphodiesterase II, and subjected to ^{32}P-postlabeling analysis using the same procedure as described previously[23] except with solvent systems for two-dimensional development. The solvent system consisted of buffer A (4.0 M lithium formate, 7.7 M urea, pH 3.5) from bottom to top, and buffer B (0.90 M lithium chloride, 0.45 M Tris-HCl, 7.7 M urea, pH 8.0) from left to right, followed by 1.7 M sodium phosphate buffer, pH 6.0, from left to right, with 3.5 cm filter paper. Adducts were detected with a Bio-Image Analyzer (BAS 3000; Fuji Photo Film, Tokyo, Japan) after exposing the TLC sheets to Fuji imaging plates. Relative adduct labeling was determined by the methods of Reddy *et al.*,[29] and values were calculated as averages using data from three assays.

Histological examination

All excised stomachs were opened along the greater curvature and washed twice with saline, then fixed in 10% neutral-buffered formalin. The fixed stomachs were sliced along the longitudinal axis into 9–12 strips of equal width, and routinely processed to sections stained with hematoxylin and eosin (H&E). The degree of chronic active gastritis was graded according to criteria modified from the Updated Sydney System,[30] by scoring the infiltration of neutrophils and mononuclear cells. Other organs, in which macroscopic lesions were observed, were also fixed in 10% neutral-buffered formalin and routinely processed to sections stained with H&E for histological examination.

Statistical analysis

The significance of differences in quantitative data for gastric inflammation, gastric adenocarcinoma and tumors of other organs was analyzed by Fisher's exact test. Data for stomach wet weight and inflammation score were examined using Tukey's multiple comparison test. Significance was concluded at $p < 0.05$.

Results

DNA adduct formation by NIAN administration in the glandular stomach of MGs

To confirm the formation of NIAN-DNA adducts in the glandular stomach of MGs, NIAN was injected two times a week at a dose of 100 mg/kg by gavage, and then analyzed by ^{32}P-postlabeling method. Three adduct spots were observed in DNA samples derived from NIAN-treated animals (Fig. 2*a*). The adduct levels were 0.3 for adduct 1, 1.1 for adduct 2, 0.2 for adduct 3 and 1.6 adducts/10^8 nucleotides

Carcinogenesis

Table 1. *H. pylori* infection induced-gastritis in MGs

Group	Treatment	Effective No.	Stomach wet weight (g)	Inflammation score
A	Broth	15	0.647 ± 0.097	0
B	NIAN + Broth	22	0.631 ± 0.094	0
C	*H. pylori*	18	1.432 ± 0.445*	2.22 ± 0.43*
D	NIAN + *H. pylori*	26	1.483 ± 0.445*	2.38 ± 0.64*

*$p < 0.01$ versus group A and B.
Values for results are expressed as averages ± SD.

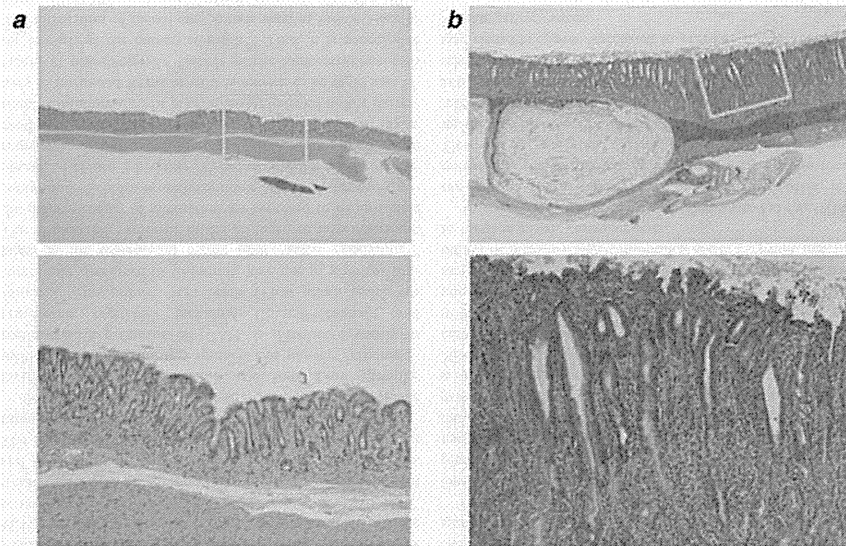

Figure 3. Macroscopic and microscopic views of gastritis in MGs infected or uninfected with *H. pylori*. (*a*) Normal gastric mucosa in group A. (*b*) Severe infiltration of many inflammatory cells with development of heterophilic proliferative glands in group C; H&E staining, ×40. Yellow boxes are shown at greater magnification below, ×200.

in total. This TLC pattern was similar to that in the *in vitro* reaction of calf thymus DNA with NIAN (total adduct level of 4.8 adducts/10^7 nucleotides, Fig. 2*b*). In the case of DNA samples derived from control animals, no adduct spots were seen on the TLC sheets (Fig. 2*c*).

Macroscopical and microscopical observation of *H. pylori*-induced gastritis in MGs

MGs were sacrificed until 104 weeks after *H. pylori* infection, and gastric disorders were analyzed. Stomach wet weights and gastric inflammation scores are shown in Table 1. Macroscopically, edematous thickening with hemorrhagic spots was apparent in the gastric mucosa in *H. pylori*-infected MGs (groups C and D), but not in animals uninfected with *H. pylori* (groups A and B). The stomach wet weight, reflecting edematous thickening, in animals infected with *H. pylori* (groups C and D) was significantly increased compared with that of animals not infected with *H. pylori* (groups A and B) ($p < 0.01$). No significant differences of stomach wet weight were detected between groups A and B and also between groups C and D.

Microscopically, gastritis, featuring infiltration of many inflammatory cells, and hyperplastic change of glandular epithelium, and erosion were observed in the pyloric regions of the animals infected with *H. pylori* (groups C and D) (Fig. 3). Heterotopic proliferative glands, whose development is related to severe gastritis in *H. pylori*-infected MGs, were sometimes observed in *H. pylori*-infected groups (groups C and D). No gastritis was found in animals not infected with *H. pylori* (groups A and B). The gastric inflammation score in *H. pylori*-infected animals was significantly increased compared with that of animals uninfected with *H. pylori* ($p < 0.01$). There were no significant differences of gastric inflammation score between groups C and D.

Table 2. Incidence of glandular stomach adenocarcinoma in MGs

Group	Treatment	Effective No.	No. of animals with glandular stomach adenocarcinoma (%) Total	Well dif.	Moderately dif.
A	Broth	15	0 (0)	0 (0)	0 (0)
B	NIAN + Broth	22	0 (0)	0 (0)	0 (0)
C	*H. pylori*	18	0 (0)	0 (0)	0 (0)
D	NIAN + *H. pylori*	26	8 (31)*	7 (27)	1 (4)

Well dif., well differentiated adenocarcinoma; Moderately dif., moderately differentiated adenocarcinoma.
*$p < 0.05$ versus group A and C and $p < 0.01$ versus group B.

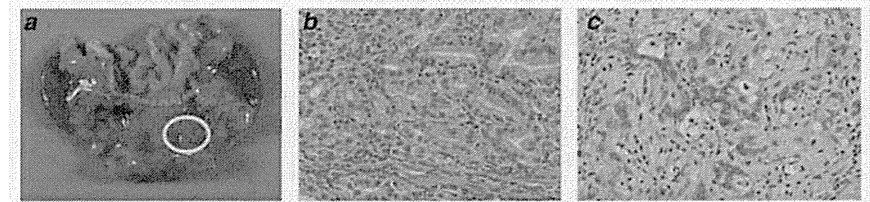

Figure 4. Histological findings of gastric adenocarcinoma in the animals treated with both NIAN and *H. pylori*. (*a*) Typical macrograph of a stomach. The yellow circle shows the suspected lesion of gastric cancer. (*b*) Well differentiated adenocarcinoma. (*c*) Moderately differentiated adenocarcinoma. (*b* and *c*) H&E staining, ×400.

Development of glandular stomach adenocarcinomas in MGs treated with both NIAN and *H. pylori*

The observed incidences of glandular stomach adenocarcinomas are shown in Table 2. Glandular stomach adenocarcinomas, histologically featuring tubular structures with cellular atypia infiltrating into the muscle layer, were found in eight animals treated with both NIAN and *H. pylori* (8/26 = 31%) at 54–104 weeks. All adenocarcinomas were observed in the pyloric mucosa and located in the lesser curvature of the stomach, where macroscopically severe edematous thickening was also seen (Fig. 4*a*). The observed adenocarcinomas in seven animals were of well differentiated (Fig. 4*b*), and a moderately differentiated lesion was observed in one animal (Fig. 4*c*). In the animals treated with broth alone, broth + NIAN and *H. pylori* alone (groups A, B and C), no glandular stomach adenocarcinomas were observed. The incidence of glandular stomach adenocarcinomas in group D was significantly higher than that in groups A, B and C ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively).

Irrespective of NIAN treatment and *H. pylori* infection, skin tumors, which histologically were well to poor differentiated squamous cell carcinomas, sebaceous carcinomas and melanomas, were found in one animal (1/15 = 7%) in group A, three animals (3/22 = 14%) in group B, two animals (2/18 = 11%) in group C and five animals (5/26 = 19%) in group D. A hemangioma was also observed in a kidney of one animal in group D (1/26 = 4%). No significant differences were apparent in these tumor incidences among groups A–D.

Discussion

In the present study, NIAN was found to induce glandular stomach adenocarcinomas in MGs in combination with *H. pylori* infection. NIAN-DNA adducts were also detected in the glandular stomach of MGs after treatment with NIAN, although clarification of their chemical structure(s) has yet to be performed. DNA adducts observed in the glandular stomachs of NIAN-treated MGs probably contain an indole-3-acetonitrile moiety. However, it is further likely that NIAN would act as an NO donor under aqueous conditions, thereby causing DNA modifications.[31–33] In fact, Lucas *et al.* demonstrated that NIAN can efficiently transfer nitroso groups to nucleophilic targets in purine nucleotides, causing *N*-nitrosation, deamination and the formation of a novel guanine analog, oxanine.[33]