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ORIGINAL ARTICLE

Relationship of sFas with metabolic risk factors and their clusters

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ABSTRACT

Background Metabolic risk factors are known to cause atherosclerosis through inflammation. In the process of inflammation, soluble Fas (sFas) may interfere with the apoptotic pathway and contribute to dysregulated inflammation. Recent studies suggest sFas as a marker of inflammation in patients with cardiovascular diseases. However, whether a relationship exists between sFas levels and metabolic risk factors among healthy subjects remains unclear.

Materials and methods We measured the serum sFas levels of 876 subjects selected as controls for a nested case–control study within the JACC Study. The adjusted means of the sFas levels were compared according to the presence of overweight/obesity, hypertension, hyperlipidaemia, diabetes and their clusters.

Results sFas level was significantly associated with overweight/obesity (2·42 ng mL^{-1} in overweight/obese men and 2·19 in others) and hyperlipidaemia (2·34 ng mL^{-1} in men with hyperlipidaemia and 2·19 in others) among men, though not with hypertension or diabetes. Moreover, a clear association between sFas levels and the cluster number of metabolic risk factors was observed independently with age, smoking and drinking(2·39, 2·28, 2·24 and 2·11 ng dL^{-1} in men with three to four, two, one and none of the four metabolic risk factors respectively). However, among women, clear associations were not observed between sFas levels and the four metabolic risk factors or their clustering.

Conclusions Serum sFas levels appear to be associated with overweight/obesity, hyperlipidaemia and clusters of metabolic risk factors among men, suggesting that sFas may elevate to down-regulate increased apoptosis in atherogenesis processes.

Keywords Metabolic risk factors, sFas.

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Introduction

Atherosclerosis has recently been recognized as an inflammatory disease linked to an abnormality of oxidation-mediated signals in the vascular system [1]. The inflammatory response plays an important role in the development of atherosclerosis, and epidemiological studies have revealed an association between inflammation and future risk for coronary heart disease [2,3]. The common risk factors for atherosclerosis, such as smoking, obesity, hypertension, hyperlipidaemia and diabetes, induce systemic oxidative stress and increase production of reactive oxygen species (ROS) [4,5]. These ROS initiate several processes involved in atherogenesis, including stimulation of vascular smooth muscle proliferation and migration, apoptosis in the endothelium and oxidation of lipids [5]. In recent studies, soluble Fas (sFas), an isoform of Fas which induces an apoptotic signal with binding to Fas ligand, was suggested to be a marker of inflammation in hyperthyroidism [6], systemic lupus erythematosus [7] and chronic kidney disease [8]. Moreover, increased sFas levels were found to predict future cardiovascular disease in patients with end-stage renal disease [9]. However, it remains unclear whether a relationship exists between sFas levels and metabolic risk factors among the general population.

We had already compared serum sFas levels of cancer mortality cases with their controls in a nested case–control study [10]. The controls we had selected in that study were from a general population but some had a history of hypertension or diabetes, and at the same time, some had anthropometric records at baseline. On the basis of such backgrounds, we investigated the possible association between metabolic risk factors drawn from such anthropometric records and sFas levels using the controls of our previous nested case–control study.

Materials and methods

Study population and serum samples

The study subjects were the controls of a nested case–control study within the Japan Collaborative Cohort (JACC) Study, a large-scale cohort study. Details of the JACC Study have been described elsewhere [11,12]. Briefly, it involved 110 792 subjects, aged 40–79 years at baseline living in 45 municipalities all over Japan. At the baseline, information on lifestyle factors was collected using self-administered questionnaires. In most areas, subjects were recruited at the time of group health check-ups and from some of them, the records of the check-ups were also collected for the study. Additionally, 35% of the cohort participants donated blood samples, and the frozen sera were kept at −80 °C until analyses were performed.

Those who died prior to the end of 1997 or suffered from cancer prior to the end of 1994 were regarded as cases of the nested case–control study. For each case, we randomly selected 3–4 controls, matching for sex, age and residential area. Eventually, 2867 cases and 10 350 controls were chosen. In this study, the subjects were selected from the controls of the nested case–control study. The study design and use of serum were approved by the Ethical Board at Nagoya University School of Medicine, where the central office of the JACC Study was located.

Definition of metabolic risk factors and their clustering

All information on lifestyle factors and medical history was obtained by self-administered questionnaires. In some areas, records were kept from health check-ups (i.e. at baseline) including height, weight, blood pressure and the levels of some serum components. Body mass index (BMI) was calculated based on the examined height and weight (BMI = weight (kg)/height $(m)^2$). We tried to define metabolic risk factors according to the modified criteria of the National Cholesterol Education Program Adult Treatment Panel III (NCEP/ATPIII) [13] as far as possible. Overweight/obesity was defined as a BMI ≥ 25 kg m^{-2}, since waist circumference was not measured in the present study. Hyperlipidaemia was defined as the state in which serum triglycerides ≥ 1·69 mmol L^{-1} (150 mg dL^{-1}) or HDL-cholesterol < 1·03 mmol L^{-1} (40 mg dL^{-1}) for men and < 1·29 mmol L^{-1} (50 mg dL^{-1}) for women. Hypertension was defined as blood pressure of ≥ 130/85 mmHg and/or when subjects reported a medical history of hypertension in their self-administered questionnaires. The subjects were defined as having diabetes when they reported a diabetes medical history since no records on serum glucose were kept. As a result, hyperlipidaemia and hypertension were defined according to the modified criteria of the NCEP/ATPIII [13], whereas the definitions of obesity and diabetes were different. Subsequently, the cluster numbers of these four metabolic risk factors (overweight/obesity, hyperlipidaemia, hypertension and diabetes) that each subject had were compiled. At the time of our baseline survey, it was not common to examine HDL-cholesterol or triglycerides. Only the subjects from six areas had the necessary information to define the metabolic risk factors mentioned above, and the analyses were restricted to these subjects.

Biochemical assays of sera

Serum levels of sFas were measured by enzyme-linked immuno-adsorbent assay (ELISA) in 1999 and 2000, using commercially available kits (MBL Co. Ltd., Nagoya, Japan). All samples were assayed at a single laboratory (SRL Inc., Hachioji, Japan) by trained staff. Assay methods have been described in detail elsewhere [14]. The range of the assay for the serum sFas level was 1·0–10 ng mL^{-1}; the intra- and inter-assay precisions were 2·1–5·5% and 8·2–12·3% respectively. For the present analysis, 928 subjects (532 men and 396 women) were eligible with data on sFas level and information on all metabolic risk factors.

Analytical method

Distributions of some baseline characteristics were compared according to the quartile of sFas levels using the Mantel-Haenszel test. Cut-off points were calculated based on the distribution of the subjects' sFas levels, men and women combined. The means of serum sFas according to the presence of metabolic risk factors and their clusters were adjusted for possible confounding factors using the analysis of covariance. Since the sFas levels had logarithmic distributions, all tests and estimations were conducted using log-transformed levels. Adjusted means of sFas were estimated (i) based on all the subjects, and (ii) based on the subjects excluding those with high sFas concentration (> 10 ng mL^{-1}, $n = 3$) and with a past history of stroke or cardiovascular disease ($n = 49$) as these conditions were suspected to elevate the sFas level. Variables adjusted in multivariate analysis were age group at baseline, area, smoking state (current smoker, ex-smoker, non-smoker and unknown), alcohol consumption (current drinkers, quitters, non-drinkers and unknown) as they were significantly associated with sFas concentration in the present study. All *P*-values were unadjusted and two-sided, and all statistical analyses were performed using the Statistical Analysis System (SAS 9·1, Cary, NC, USA).

Results

Table 1 shows the distribution of baseline characteristics according to sFas quartiles (≤ 1·8, 1·8–2·1, 2·1–2·4 and > 2·4 ng mL^{-1}). Those with higher sFas levels were statistically older than those with lower levels. Among men, smokers tended to be observed more and drinkers tended to be observed less among higher sFas levels. Though not significant, the men in the top quartile were walking longer and highly educated compared to other men. Similarly, women with higher sFas levels were less likely to eat green leafy vegetables compared to those with lower levels, although education showed the opposite trend to that of men.

Adjusted means of sFas according to the presence of metabolic risk factors and their clusters are shown in Table 2. Overweight/obesity and hyperlipidaemia were statistically associated with increased sFas levels; however, hypertension and diabetes did not show any association with sFas levels among men. Among women, none of these four factors were found to be associated with sFas levels. Clustering numbers of these four metabolic risk factors were clearly statistically associated with sFas levels independent of age, smoking and drinking among men. The adjusted means of the sFas levels increased according to the increase in the cluster numbers, and the top category (presence of 3–4 metabolic risk factors) showed a statistically higher level compared with the lowest category (without any of these risk factors: 2·39 ng mL^{-1} vs. 2·11 ng mL^{-1}). However, no association was observed among women between sFas levels and clustering numbers of four metabolic risk factors, although an increasing trend did exist except in the lowest category. Excluding the subjects with high sFas levels (> 10 ng mL^{-1}) and with a past history of stroke or cardiovascular disease did not alter the result.

Discussion

We found that the sFas level was statistically significantly associated with overweight/obesity and hyperlipidaemia among men, although not with hypertension or a history of diabetes.

Table 1 sFas levels and some baseline characteristics

	sFas level (ng mL^{-1})					
	≤ 1·8	1·8–2·1	2·1–2·4	> 2·4	Total	*P*-value*
Men						
Mean age at baseline†	60·5	61·2	62·3	63·8	61·9	< 0·01
Current cigarette smoker (%)	41·0	43·3	50·5	47·7	45·3	0·01
Current alcohol drinker (%)	79·7	74·4	68·2	60·6	71·0	< 0·001
Walking ≥ 1 h day^{-1} (%)	30·6	27·0	31·2	35·6	30·9	
College or higher education (%)	13·3	13·2	17·8	22·0	16·4	
Eating green vegetables almost daily (%)	28·7	31·6	35·0	30·4	31·1	
Total number	153	133	110	136	532	
Women						
Mean age at baseline†	56·6	61·8	62·5	64·1	61·6	< 0·0001
Current cigarette smoker (%)	5·0	3·7	6·5	3·4	4·7	0·09
Current alcohol drinker (%)	27·1	17·9	15·8	15·7	20·4	
Walking ≥ 1 h day^{-1} (%)	24·1	39·3	36·7	35·3	31·8	
College or higher education (%)	7·4	7·7	4·1	5·0	6·3	
Eating green vegetables almost daily (%)	34·6	40·4	35·4	30·0	35·1	
Total number	145	84	78	89	396	

Each lifestyle factor has missing values, and total is not 100%.
*Adjusted for age groups and residual area.
†Adjusted for residual area.

Table 2 Adjusted means of sFas and metabolic risk factors

	Total subjects						Subjects excluding those with high sFas level or a disease history					
	N	%	Adjusted means of sFas (ng mL^{-1})	95% CI		*P*-value*	*N*	%	Adjusted means of sFas (ng mL^{-1})	95% CI		*P*-value*
Men												
Overweight/obesity												
Absence	395	74·2	2·19	2·06	2·33	< 0·001	368	74·6	2·21	2·09	2·34	< 0·01
Presence	137	25·8	2·42	2·25	2·60		125	25·4	2·40	2·25	2·56	
Hypertension												
Absence	129	24·2	2·24	2·08	2·41	0·71	122	24·7	2·21	2·07	2·35	0·13
Presence	403	75·8	2·26	2·13	2·41		371	75·3	2·29	2·17	2·42	
Hyperlipidaemia												
Absence	336	63·2	2·19	2·06	2·34	0·01	316	64·1	2·22	2·09	2·35	0·03
Presence	196	36·8	2·34	2·19	2·50		177	35·9	2·33	2·20	2·48	
Diabetes												
Absence	488	91·7	2·26	2·13	2·41	0·50	453	91·9	2·27	2·15	2·40	0·73
Presence	44	8·3	2·20	1·98	2·43		40	8·1	2·24	2·05	2·45	
Number of metabolic risk factors												
			Trend P = 0·03						Trend P = 0·01			
0	82	15·4	2·11	1·95	2·29	Ref.	80	16·2	2·13	1·99	2·29	Ref.
1	213	40·0	2·24	2·09	2·40	0·12	198	40·2	2·25	2·11	2·40	0·10
2	148	27·8	2·28	2·13	2·45	0·04	134	27·2	2·28	2·14	2·42	0·06
3–4	89	16·7	2·39	2·21	2·59	< 0·01	81	16·4	2·41	2·25	2·59	< 0·01
Total	532	100·0					493	100·0				
Women												
Overweight/obesity												
Absence	277	69·9	2·17	1·91	2·47	0·67	268	70·0	2·17	1·92	2·45	0·42
Presence	119	30·1	2·20	1·93	2·51		115	30·0	2·22	1·96	2·51	
Hypertension												
Absence	116	29·3	2·19	1·91	2·51	0·87	114	29·8	2·21	1·94	2·51	0·70
Presence	280	70·7	2·18	1·92	2·47		269	70·2	2·18	1·94	2·46	
Hyperlipidaemia												
Absence	194	49·0	2·19	1·92	2·49	0·87	187	48·8	2·18	1·93	2·46	0·78
Presence	202	51·0	2·18	1·92	2·47		196	51·2	2·19	1·94	2·47	
Diabetes												
Absence	384	97·0	2·18	1·92	2·47	0·98	372	97·1	2·19	1·94	2·46	0·92
Presence	12	3·0	2·19	1·80	2·65		11	2·9	2·20	1·83	2·66	

Table 2 *Continued*

	Total subjects						Subjects excluding those with high sFas level or a disease history					
	N	%	Adjusted means of sFas (ng mL^{-1})	95% CI		*P*-value*	*N*	%	Adjusted means of sFas (ng mL^{-1})	95% CI		*P*-value*
Number of metabolic risk factors												
			Trend P = 0·97						Trend P = 0·75			
0	54	13·6	2·21	1·91	2·56	Ref.	52	13·6	2·23	1·94	2·56	Ref.
1	143	36·1	2·17	1·90	2·47	0·63	140	36·6	2·15	1·90	2·44	0·39
2	131	33·1	2·19	1·92	2·49	0·79	126	32·9	2·19	1·94	2·48	0·71
3–4	68	17·2	2·19	1·91	2·51	0·86	65	17·0	2·22	1·95	2·53	0·98
Total	396	100·0					383	100·0				

Adjusted for age group, area, smoking status and drinking status.
Overweight/obesity; BMI ≥ 25 kg m^{-2}, Hypertension; ≥ 130/85 mmHg and/or medical history of hypertension, Hyperlipidaemia; serum triglycerides ≥ 1·69 mmol L^{-1} (150 mg dL^{-1}) or HDL-cholesterol < 1·03 mmol L^{-1} (40 mg dL^{-1}) for men and < 1·29 mmol L^{-1} (50 mg dL^{-1}) for women, Diabetes; medical history of diabetes.

Moreover, a clear association between sFas levels and the cluster number of metabolic risk factors was independently observed with age, smoking and drinking. In contrast, among women, we failed to find any clear association between the four metabolic risk factors and sFas levels.

Hebert *et al.* showed that plasma levels of sFas were associated with coronary artery disease in stable patients with end-stage renal disease [15]. Recently, a study examined plasma sFas levels in patients with coronary heart disease (CHD), CHD-equivalent, or 10-year CHD risk > 20% [16]. sFas levels were found to be statistically higher in such patients compared to healthy subjects as a result of our study. Moreover, they demonstrated that the sFas concentration was related to different cardiovascular risk factors such as diabetes, metabolic syndrome, and hypertension, and short-term treatment with atorvastatin, which has an anti-inflammatory effect, lowered sFas concentrations in these patients. These results suggest that sFas may play an important role in inflammation processes at atherosclerotic lesions. Another prospective study on dialysis patients also revealed that increased plasma sFas levels were significantly associated with future cardiovascular endpoints [9], suggesting that sFas is a novel predictor of active atherosclerotic disease.

Metabolic disorders, such as obesity, hypertension, hyperlipidaemia and diabetes are well-known risk factors for cardiovascular disease [17–19]. They increase production of ROS and initiate the process of atherosclerosis [5]. Endothelial injury or exposure to ROS induces apoptosis of endothelial cells, which leads to endothelial cell loss and results in atherogenesis and a procoagulative state [1]. The Fas–FasL system is one of the important death factors causing apoptosis to cells [20]. In contrast, sFas, a splicing variant of Fas, binds FasL and acts as a competitive antagonist of Fas apoptotic signalling [21]. Thus, sFas may interfere with the apoptotic pathway in the process of atherosclerosis and contribute to dysregulated inflammation [22].

Gender differences were observed in the association of metabolic risk factors and sFas levels; a positive association was found in men and no association in women. Most prior studies on atherosclerosis or metabolic disorders and sFas levels did not show results separated by gender [9,15,16]. Our cross-sectional study was not designed to determine the mechanisms of the gender differences. However, the result suggests that gender differences exist in the process of atherosclerosis through inflammatory response. For example, inflammation measured by C-reactive protein (CRP) levels showed a weaker effect on mortality from cardiovascular disease in women than in men [3]. Another study found that atherosclerosis, measured by common carotid artery intima-media thickness, was related to CRP in men, but not in women [23]. These gender differences deserve further attention.

Some limitations of our study must be discussed. First, the definitions we used to categorize metabolic risk factors were somewhat different from the diagnostic criteria adopted today [13,24]. This is because we did not obtain some of the necessary information at the baseline survey. However, except for diabetes, our definitions were the very same as those adopted in a recent Japanese study [19]. The BMI level of ≥ 25 kg m^{-2}, used in this paper instead of waist circumference, was reported to correspond well to the Asian criterion for high waist

circumference of ≥ 90 cm in men and ≥ 80 cm in women [25]. Diabetes was defined based on a self-report as we had no records on serum fasting glucose. Self-reported medical histories were found to be accurate among Japanese workers [26]. In the US, among residents ≥ 45 years of age, substantial agreement was found between self-reports and medical records (κ = 0·71–0·80) for diabetes [27]. Thus, the metabolic risk factors we adopted in this paper likely indicate overweight/obesity, hypertension, hyperlipidaemia, and diabetes, accurately, and we found a clear association of sFas level with clusters of these four metabolic risk factors among men. Second, since not all the cohort participants provided blood samples, there was the possibility of selection bias. Further, HDL-cholesterol and triglyceride were not commonly examined at the time of the baseline survey, and the number of subjects we analysed was relatively small compared to the original controls of the nested case–control study. However, donation depended solely on the subject's intention, and the control selection in the nested case–control study was based only on matching information. The examination of HDL-cholesterol and triglyceride depended on the health check-up system of each area. Thus, any bias due to blood donation or subject selection should not seriously affect our results. Third, serum samples were stored for approximately 10 years at −80 °C. The stability of sFas in these cohort samples could not be determined because their levels were not measured at baseline. However, Ito *et al.* compared newly collected sera and frozen specimens stored for 9 years gathered from a variety of different individuals, and found no statistically significant difference in the distributions of sFas levels [14], indicating that the serum sFas level remained stable after long-term storage at −80 °C.

In conclusion, serum sFas levels appear to be associated with overweight/obesity, hyperlipidaemia and cluster of metabolic risk factors among men, suggesting that serum sFas may elevate to down-regulate increased apoptosis in atherogenesis processes.

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ORIGINAL PAPER

Evaluation of Onco*type* DX Recurrence Score as a prognostic factor in Japanese women with estrogen receptor-positive, node-negative primary Stage I or IIA breast cancer

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Abstract
Purpose We sought to evaluate the use of the **Onco*type*** DX Breast Cancer Assay for identifying candidates for adjuvant therapy in patients with estrogen receptor (ER)-positive, node-negative primary Stage I or IIA breast cancer.
Methods A retrospective case–control study was conducted on 40 patients who underwent surgery between 2000 and 2008. Cases ($n = 10$) were patients who had metastases after surgery. Controls ($n = 30$) were patients who did not develop metastases and were individually matched to their case with respect to age. All patients were analyzed with regard to age, tumor size, histological grade, HER2 status, and the values of Recurrence Score (RS), ER score and PgR score generated by **Onco*type*** DX. We also divided the patients into low, intermediate or high-risk groups according to individual RS values.
Results RS, risk category and histological grade were associated with metastases in patients with ER-positive, node-negative Stage I or IIA breast cancer. However, ER status, tumor size and PgR status were not associated with metastases. Histological grade was associated with RS value and the distribution pattern of risk category ($P < 0.001$ for each).
Conclusions Both histological grade and risk-category classification were effective in identifying women at risk of developing distant metastases after initial therapy for ER-positive, node-negative Stage I or IIA breast cancer. These patients may benefit from the addition of adjuvant therapy at diagnosis.

Keywords Oncotype DX · Japanese women · ER positive · Node negative · Adjuvant therapy · Breast cancer

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Introduction

More than 40,000 women are newly diagnosed with breast cancer each year in Japan, and the number of patients with breast cancer has been increasing. Breast cancer became the most common cancer in women in 1994, and it is the most common cause of death among women between the ages of 30 and 64 in Japan. About one-third of patients with breast cancer develop distant metastases after the initial surgical resection (Kamo et al. 2008; Matsuda et al. 2008), and this includes some women with estrogen receptor (ER)-positive, node-negative Stage I or IIA breast cancer. It is extremely difficult to predict the risk of recurrence for this patient population, and the **Onco*type*** DX system was developed to facilitate prognostic determination. **Onco*type*** DX is a 21-gene assay that calculates the Recurrence Score (RS), a statistic designed to predict the risk of distant recurrence during a 10-year period, and predict the response to chemotherapy for lymph node-negative, hormone-positive breast cancer (Wolf et al. 2008). It uses reverse transcriptase-polymerase chain reaction (**Onco*type*** DX Breast Cancer Assay, Genomic Health, Inc, Redwood City, CA, USA) to calculate an individualized RS using a proprietary algorithm. The RS has been prospectively validated as a predictor of 10-year recurrence-free survival in patients with node-negative, ER-positive early stage breast cancer (Paik et al. 2004).

However, most of the studies examining the **Onco*type*** DX system have been conducted in the USA and Europe among Caucasian women, but the genetic background of patients with breast cancer can differ dramatically by race (Balan et al. 2008). This may have profound impacts on the clinical course and response to treatment. The purpose of this case–control study was to evaluate the utility of Onco*type* DX in identifying Japanese women with ER-positive, node-negative Stage I or IIA primary invasive breast cancer who were candidates for adjuvant therapy.

Patients and methods

Patients

A case–control study was conducted under informed consent among 40 patients diagnosed with ER-positive, node-negative Stage I or IIA primary breast cancer who underwent surgery from January 2000 to December 2008 in Aichi Medical University and Marumo Hospital. Cases ($n = 10$) were consecutive patients who had distant breast cancer metastases after surgery. Controls ($n = 30$) were patients who did not have metastatic lesions during the observation period and who were individually matched to their case with respect to age (within 2 years). Patients underwent adjuvant hormone therapy or chemotherapy at the discretion of their individual doctors, and patients were excluded from the study if they had evidence of mucinous adenocarcinoma with a probability of a good prognosis.

Patient evaluation

Patients were analyzed with regard to such classical clinicopathologic features as age, tumor size, histological grade, HER2 status, lymphatic invasion and vascular invasion. All immunohistochemistry and histological determinations were performed by a single pathologist. We also analyzed the RS, the ER score and the PgR score generated by **Onco*type*** DX Breast Cancer Assay for each patient. The ER score and the PgR score are included in the calculation of the RS. All of the patients were then classified into three groups based on the RS value as follows: low (RS < 18), intermediate (RS 18–30) or high (RS ≥ 31) risk-category groups. We compared the clinicopathologic characteristics of the case and control patients. The patients underwent the following evaluations every year: physical examination, blood tests, mammography, breast ultrasonography and computed tomography. The length of the follow-up period was calculated from the date of the first surgery to the date of the last visit to our outpatient clinic, and the presence of metastasis, death and cause of death were recorded for all appropriate patients.

Statistical analysis

All analyses were conducted using SPSS software, version 9.0. All confidence intervals (CI) are reported as 95% CI. Data are reported as the mean ± SD. When possible, all tests were two-sided, and $P < 0.05$ was considered significant for all analyses. Categorical variables were compared using the Fisher's exact test or Spearman's rank correlation coefficient. Continuous variables were compared using Student's *t* test. We conducted multivariable logistic regression analysis to determine whether RS or histological grade were associated with distant metastasis.

Onco*type* DX

For eligible cases and controls, the **Onco*type*** DX assay (Genomic Health Institute, Redwood City, CA, USA) was performed on preserved paraffin blocks according to the manufacturer's instructions.

Results

Table 1 lists the patient and tumor characteristics of the study population. All of the patients included in this study underwent surgery with an axillary dissection or a sentinel node biopsy. Among the cases ($n = 10$), two patients underwent breast conserving surgery, and eight patients underwent mastectomy. For the controls ($n = 30$), 19 patients had breast conserving surgery and 11 underwent mastectomy ($P = 0.028$). One of the case patients was given chemotherapy (CEF six courses) and four control patients had chemotherapy. These patients were all given different treatment regimens as follows: CMF three courses, EC four courses, CEF four courses and DOC four courses, and CMF six courses. There were no statistically significant differences between the cases and controls for the following parameters:

Table 1 Study population

	Cases	Controls	*P* value
No. of patients	10	30	
Age (year)	49.1 ± 12.9 (37–76)	50.9 ± 11.9 (37–78)	0.693
Tumor size (mm)	18.9 ± 4.6 (95% CI 15.7–22.2)	15.8 ± 6.3 (95% CI 13.4–18.2)	0.165
T1	6 (60%)	23 (77%)	0.418
T2	4 (40%)	7 (23%)	
Histological grade			
1	1 (10%)	15 (50%)	0.001
2	1 (10%)	9 (30%)	
3	8 (80%)	5 (17%)	
Unknown	0 (0%)	1 (3%)	
HER2 status			
Negative	7 (70%)	27 (90%)	0.057
Positive	2 (20%)	0 (0%)	
Unknown	1 (10%)	3 (10%)	
ly			
Positive	1	2	1.000
Negative	9	28	
v			
Positive	0	0	
Negative	10	30	
ER score	9.1 ± 1.7 (95% CI 7.9–10.3)	10.1 ± 1.3 (95% CI 9.6–10.5)	0.068
PgR score	6.6 ± 2.0 (95% CI 5.2–8.0)	7.7 ± 1.5 (95% CI 7.1–8.3)	0.069
RS	40.0 ± 26.4 (95% CI 21.1–58.9)	17.8 ± 10.9 (95% CI 13.8–21.9)	<0.001
Risk category group			
Low risk	3 (30%)	19 (63%)	0.005
Intermediate risk	1 (10%)	8 (27%)	
High risk	6 (60%)	3 (10%)	
Procedure performed			
Mastectomy	8 (80%)	11 (37%)	0.028
Partial mastectomy	2 (20%)	19 (63%)	
Adjuvant therapy			
Hormone therapy	9 (90%)	26 (87%)	0.999
Chemotherapy	1 (10%)	4 (13%)	

age at diagnosis, tumor size, HER2 status, lymphatic invasion, vascular invasion, the ER score, the PgR score and the type of the adjuvant therapy. The median follow-up period was 53.4 months for the cases and 55 months for the controls. When the cases were compared to the controls, the mean RS value of the cases [40.0 (95% CI 21.1–58.9)] was significantly ($P < 0.001$) higher than that of the controls [17.8 (95% CI 13.8–21.9)], and the proportion of patients in the RS risk categories also differed ($P = 0.005$) such that more cases were classified as high risk. Finally, there were significant differences between the case and control patients in the tumor histological grades ($P = 0.001$), and more cases had a histological grade 3 tumor than control patients.

We then examined the patients with a histological grade 3 tumor ($n = 13$) in greater detail and found a significant difference only in the distribution of the frequency of the type of adjuvant therapy between the patients with metastases ($n = 8$) and those without metastases ($n = 5$; $P = 0.035$; Table 2). However, when a similar analysis was performed with the patients stratified by RS-based high-risk category, a marginally significant difference was seen in treatment regimen ($P = 0.083$; Table 3).

Table 4 lists the characteristics of the patients treated with adjuvant hormone therapy. There were significant differences in RS ($P < 0.001$), risk category group ($P < 0.001$), histological grade ($P = 0.003$) and the PgR score ($P = 0.034$) between the patients with metastases ($n = 9$) and those without metastases ($n = 26$).

Among the patients with metastatic disease, four patients died as a result of progression of their metastatic breast

Table 2 Adjuvant therapy for patients with histological grade 3 tumor

	Cases	Controls	*P* value
No. of patients	8	5	
Adjuvant therapy			
Hormone therapy	8	2	0.035
Chemotherapy	0	3	

Table 3 Adjuvant therapy for patients classified into high risk group

	Cases	Controls	*P* value
No. of patients	6	3	
Adjuvant therapy			
Hormone therapy	6	1	0.083
Chemotherapy	0	2	

cancer, and the first site of metastasis for each patient is shown in Table 5. The median disease free survival period for these patients was 17 months. Among the four patients who died of metastatic disease, the median interval to recurrence was 18 months, and the median time from surgery to death was 56 months.

We also examined the distribution of the RS value and the distribution of RS category according to histological grade for all of the patients. Histological grade was strongly associated with the RS value ($P < 0.001$; Fig. 1) and the distribution of RS category ($P < 0.001$, $\rho = 0.63$; Table 6).

Multivariable logistic regression analysis was performed to explore the relation between distant metastasis and age, ER score, PgR score, RS, histological grade and lymphatic invasion. Histological grade 3 was significantly associated with distant metastasis (Table 7).

Discussion

The prognosis for the patients with ER-positive, node-negative Stage I or IIA breast cancer is better than that for patients with more advanced invasive breast cancer, but, even within this low-stage group, some patients experience disease recurrence or metastasis and ultimately die of metastatic disease. It remains difficult to reliably predict the prognosis of patients with ER-positive, node-negative Stage I or IIA breast cancer, but the Onco*type* DX system was developed to assist in prognostic determination of this patient group. Some studies have estimated the risk category for patients, and this has been incorporated into treatment decisions (i.e., the need for adjuvant therapy) for patients with invasive breast cancer in the United States. However, no one has examined the utility of using RS in the treatment algorithm of women with invasive breast cancer in Japan. The RS incorporates several different biological parameters into its calculation, and it is possible that RS may not have the same degree of accuracy in patients with different genetic background.

We stratified the patients into three risk categories based on their RS, and found a significant difference in histological grades for each risk category between patients with and without metastases. Furthermore, patients with both metastases and a tumor of histological grade 3 ($n = 8$), were significantly more likely to have received hormone therapy than patients with a histological grade 3 tumor who remained cancer free ($n = 5$; $P = 0.035$). However, when patients with ($n = 6$) or without ($n = 3$) metastases were classified into the high-risk category based on their RS, there was a marginally significant difference in the type of adjuvant therapy used ($n = 3$; $P = 0.083$). Our sample size

Table 4 Patient characteristics treated with hormone therapy

	Cases	Controls	*P* value
No. of patients	9	26	
Age (year)	50.0 ± 13.4 (37–76)	52.3 ± 11.9 (37–78)	0.636
Tumor size	18.4 ± 4.5 (95% CI 14.9–21.8)	15.8 ± 6.4 (95% CI 13.2–18.5)	0.28
Histological grade			
1	1 (10%)	15 (58%)	0.003
2	1 (10%)	8 (31%)	
3	8 (80%)	2 (8%)	
Unknown	0 (0%)	1 (4%)	
RS	42.9 ± 26.3 (95% CI 22.7–63.1)	16.2 ± 10.3 (95% CI 12.0–20.4)	<0.001
Risk category group			
Low risk	2 (22%)	18 (69%)	<0.001
Intermediate risk	1 (11%)	7 (27%)	
High risk	6 (67%)	1 (4%)	
ER score	9.1 ± 1.8 (95% CI 7.7–10.5)	10.2 ± 1.4 (95% CI 9.6–10.7)	0.073
PgR score	6.5 ± 2.1 (95% CI 4.9–8.1)	7.8 ± 1.4 (95% CI 7.3–8.4)	0.034

Table 5 Case characteristics

Characteristic	Patient no.									
	1	2	3	4	5	6	7	8	9	10
Age at diagnosis (year)	41	37	64	76	55	41	37	53	47	40
Size (mm)	24	18.2	18	27	16	15	21	21	18	11
Histological grade	2	3	3	1	3	3	3	3	3	3
HER2	1+	1+	1+	–	3+	–	3+	1+	–	Unknown
ER score	8.88	10.1	9.71	11.81	9.33	8.03	6.24	7.11	8.8	10.84
PgR score	7.73	8.2	5.42	9.46	2.99	6.51	5.09	4.89	7.27	8.42
Recurrence Score	14	37	32	12	75	20	72	74	52	12
Risk category group	1	3	3	1	3	2	3	3	3	1
Adjuvant therapy	C	H	H	H	H	H	H	H	H	H
Interval from surgery to recurrence (month)	37	12	29	5	62	54	18	18	52	43
The first site of metastasis	Bone	Brain	Lung	Bone	Brain	Bone	Lung	LN	Lung	Lung
Outcome	Alive	Alive	Alive	Dead	Alive	Alive	Dead	Dead	Alive	Dead
Interval from surgery to death (month)				108			48	27		64

C chemotherapy, *H* hormone therapy

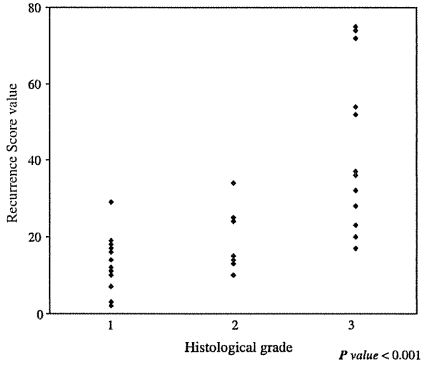

Fig. 1 The distribution of the RS value according to the histological grade. $P < 0.001$

was likely too small, though, to detect any significant differences for this parameter.

We found significant differences in the proportion of the tumor histological grade ($P = 0.001$) and the RS risk category ($P = 0.005$) and the mean RS value ($P < 0.001$) between the case and control patients. This data suggest the tumor histological grade is stronger prognostic power than the RS risk category. However, biopsies and even surgical specimens are subject to sampling and pathologist error, and there can be also interobserver disagreement in nuclear atypia scoring of node-negative breast cancers (Tsuda et al. 1999). By relying upon biochemical and genetic markers of disease, RS generated by **Oncotype** DX is objectively

Table 6 The distribution of the risk category group according to the histological grade

Risk category group	Histological grade		
	1	2	3
Low risk	13	7	2
Intermediate risk	3	2	3
High risk	0	1	8

$P < 0.001$, $\rho = 0.63$

Table 7 Multivariate logistic regression analysis of age, ER score, PgR score, RS, histological grade, ly in relation to the likelihood of distant metastasis

Variable	*P* value	Odds ratio (95% CI)
Age at diagnosis	0.195	0.90 (0.764–1.057)
ER score	0.651	1.33 (0.389–4.53)
PgR score	0.378	0.65 (0.246–1.702)
RS 50 or more vs RS less than 50	0.579	2.85 (0.07–115.552)
Histological grade 2 vs histological grade 1	0.369	7.48 (0.093–602.504)
Histological grade 3 vs histological grade 1	0.041	222.0 (1.243–39647.336)
ly(+) vs ly(−)	0.557	0.37 (0.013–10.312)

determined and independent of the skill and diagnostic acumen of pathologists. Additionally, these biomarkers may better reflect cancer pathophysiology than morphology alone. Our data also suggest that the RS value has a stronger prognostic power than the tumor histological grade. It seems to be valid to regard the RS value, with a universal

validity, as one of the strongest prognostic power in determining adjuvant therapy for Japanese female patients with ER-positive, node-negative Stage I or IIA breast cancer.

Consistent with this, several patients in this study stratified to the low risk category or with a histological grade 1 tumor eventually experienced metastasis. Thus, factors that are not apparent on biopsy or incorporated into the RS may strongly affect the tendency for breast cancer to metastasize. Additionally, the tumor region sampled for processing by **Oncotype** DX may affect the assignment of risk category and the prognosis of the disease. As a tumor expands, the genetic instability inherent in malignancy may give rise to a heterogeneous population of cells. Tissue sampled from one region may not fully reflect the malignant potential of the tumor as a whole, and the calculated RS may inappropriately place the patient in a lower-risk category. Future studies are needed to determine the location and number of samples submitted for Oncotype DX analysis.

We conducted multivariable logistic regression analysis to determine whether RS or histological grade were associated with distant metastasis among the Japanese female patients with ER-positive, node-negative, Stage I or IIA breast cancer. The results from multivariable logistic regression analysis showed that histological grade was strongly associated with metastasis. Although statistically insignificant, high values of RS was also associated with the recurrence, with OR being 2.85 (95% CI 0.07–115.5), after adjustment for age, ER, PgR, ly and histological grade. The OR of the RS for distant metastasis was similar to the data shown by Paik et al. (2004). Due to the small sample size, the overall risk estimation from logistic regression analysis in our study was unstable with wide 95% CI.

Oncotype DX analysis is not frequently covered by insurance in Japan, and if patients are not able to afford **Oncotype** DX studies, the histological grade may be an adequate surrogate.

This case–control study of 40 Japanese women with ER-positive and node-negative primary breast cancer is one of the largest series in Japan, but there are several caveats to this study. In particular, the number of the patients involved was small and the use and the type of adjuvant therapy differed between patients. Prospective studies should be conducted using RS as a strategy to assign women to conventional or adjuvant therapy in the Japanese population, and such studies will more definitively establish if there is a role for RS in therapeutic decision making for ER-positive, node-negative Stage I or IIA breast cancer.

In conclusion, we examined the prognostic value of the RS calculated by **Oncotype** DX in Japanese female patients with ER-positive, node-negative Stage I or IIA breast cancer. This is the first case–control study demonstrating that increased RS value is associated with disease recurrence in this patient population. Our study suggests that patients with a tumor of histological grade 3 or an increased RS should be considered for adjuvant chemotherapy at diagnosis because they have a higher degree of metastasis.

Conflict of interest statement We declare that we have no conflict of interest.

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BMI and All-cause Mortality Among Japanese Older Adults: Findings From the Japan Collaborative Cohort Study

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The association between BMI and all-cause mortality may vary with gender, age, and ethnic groups. However, few prospective cohort studies have reported the relationship in older Asian populations. We evaluated the association between BMI and all-cause mortality in a cohort comprised 26,747 Japanese subjects aged 65–79 years at baseline (1988–1990). The study participants were followed for an average of 11.2 years. Proportional-hazards regression models were used to estimate mortality hazard ratios (HRs) and 95% confidence intervals. Until 2003, 9,256 deaths occurred. The underweight group was associated with a statistically higher risk of all-cause mortality compared with the mid-normal-range group (BMI: 20.0–22.9); resulting in a 1.78-fold (95% confidence interval: 1.45–2.20) and 2.55-fold (2.13–3.05) increase in mortality risk among severest thin men and women (BMI: <16.0), respectively. Even within the normal-range group, the lower normal-range group (BMI: 18.5–19.9) showed a statistically elevated risk. In contrast, being neither overweight (BMI: 25.0–29.9) nor obese (BMI: ≥30.0) elevated the risk among men; however among women, HR was slightly elevated in the obese group but not in the overweight group compared with the mid-normal-range group. Among Japanese older adults, a low BMI was associated with increased risk of all-cause mortality, even among those with a lower normal BMI range. The wide range of BMI between 20.0 and 29.9 in both older men and women showed the lowest all-cause mortality risk.

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INTRODUCTION

The relationship between high BMI (BMI: weight in kg/height in m²) and all-cause mortality is well known (1,2). The World Health Organization defines overweight as a BMI of 25.0–29.9 kg/m² and obesity as a BMI of ≥30 kg/m². These BMI thresholds have been recommended worldwide for all individuals aged ≥18 (3). However, increasing evidence suggests that the association between BMI and mortality varies with age. A 2007 review by Janssen and Mark concluded that BMIs in the overweight range (BMI: 25.0–29.9) were not associated with a significant increase in mortality risk among the older adults (4). Furthermore, some recent studies have revealed that among this age group, being underweight seems to be a better predictor of mortality than obesity (5–7). Thus, it remains to be established whether older adults require different BMI cut-off points from those younger.

Japan has witnessed a rapid growth in its older population in recent years. From a public health perspective, it is important to determine the BMI range associated with a low mortality risk for them. We sought to examine the association between BMI and all-cause mortality among participants in our Japan Collaborative Cohort study.

METHODS AND PROCEDURES

Study subjects and data collection

The study design and methods adopted by the Japan Collaborative Cohort study have been previously described elsewhere (8,9). Briefly, from 1988 to 1990, healthy subjects in 45 areas throughout Japan replied to a self-administered questionnaire. The cohort comprised 110,792 subjects aged 40–79 years old at baseline, among whom those participants aged 65–79 years were enrolled in this study. The ethical board of the Nagoya University School of Medicine, where the central office of the Japan Collaborative Cohort study was located, has approved our complete study design.

Follow-up

The cause and date of death of the study subjects were identified by reviewing all death certificates in each area by each area investigator with the permission of the Director-General of the Prime Minister's Office (Ministry of Internal Affairs and Communications). Those who had moved out of a study area were treated as censored. Follow-ups were conducted to the end of 2003, except in four areas where they were discontinued at the end of 1999.

BMI

Information on height and weight as well as lifestyle variables was gathered from self-administered questionnaires. BMI at baseline was calculated based on the height and weight reported. We grouped subjects into the following nine detailed categories according to the World Health Organization classification (10): BMIs <16.0, 16.0–16.9, 17.0–18.4, 18.5–19.9, 20.0–22.9, 23.0–24.9, 25.0–27.4, 27.5–29.9, and ≥30.0. These categories incorporated the current definitions of underweight (BMI: <18.5), normal range (18.5–24.9), overweight (25.0–29.9), and obese (≥30.0) (3). There were 26,747 subjects (11,230 men and 15,517 women) aged 65–79 years who provided information on BMI, all of whom were considered to be eligible for this study.

Analysis

To compare the proportions of subject characteristics across BMI categories at baseline, we used the Mantel–Haenszel test. Hazard ratios (HRs) were calculated separately by gender according to Cox's proportional hazard model. Not only in all the subjects combined but also in subcohorts of noncurrent smokers, physically active subjects (engaging in physical exercise ≥1 h per week and/or walking >1 h/day), and those

Table 1 Distribution of some demographic factors according to BMI categories

		BMI category								
		<16.0	16.0–16.9	17.0–18.4	18.5–19.9	20.0–22.9	23.0–24.9	25.0–27.4	27.5–29.9	≥30.0
Men										
Age at baseline										
65–69	%	19.0	31.0	36.5	43.8	47.4	49.5	52.7	51.6	50.6***
70–74	%	34.1	30.6	37.4	32.8	32.1	32.5	32.8	31.2	28.6
75–79	%	46.8	38.4	26.1	23.4	20.5	18.0	14.4	17.2	20.8
Current cigarette smoker	%	50.8	48.7	52.8	47.2	42.6	34.3	34.2	30.8	29.9***
Current alcohol drinker	%	44.4	53.9	56.5	60.7	62.0	61.4	61.8	66.7	50.6***
Sleep 6.5–8.4 h/day	%	49.2	57.3	54.0	57.4	59.8	60.8	57.7	54.8	48.1*
Physically active	%	42.9	47.4	46.7	48.9	49.6	47.6	44.8	40.9	42.9
College or higher education	%	11.1	12.1	11.9	12.6	13.6	14.6	14.8	12.2	9.1
High-mental stress	%	7.9	13.8	9.8	8.7	7.8	8.6	7.7	10.0	11.7*
Married	%	66.7	75.9	70.6	72.6	73.7	76.4	77.3	80.3	75.3*
Eating green vegetables almost daily	%	23.0	31.0	28.1	30.8	29.3	28.8	27.8	23.7	15.6*
No prior disease history (cancer, MI, or stroke)	%	46.0	59.5	59.5	62.7	65.8	63.3	64.8	62.7	66.2***
Number		126	232	871	1,622	4,670	2,217	1,136	279	77
Women										
Age at baseline										
65–69	%	28.1	41.7	43.0	45.4	50.0	54.5	56.7	58.2	53.2***
70–74	%	37.6	32.8	33.4	31.8	31.3	29.8	29.2	28.6	28.4
75–79	%	34.3	25.5	23.5	22.9	18.7	15.7	14.1	13.2	18.4
Current cigarette smoker	%	7.9	9.0	6.0	4.7	3.6	3.5	3.8	5.6	5.1***
Current alcohol drinker	%	11.6	14.2	16.3	16.3	16.3	17.7	15.9	18.1	15.1
Sleep 6.5–8.4 h/day	%	50.0	54.2	55.6	54.7	58.0	56.0	56.6	53.3	54.7*
Physically active	%	29.8	40.9	42.4	43.7	45.4	44.0	42.9	38.9	35.0***
College or higher education	%	9.5	4.9	6.8	6.2	6.2	5.6	5.0	3.8	4.2***
High-mental stress	%	14.5	7.5	10.4	9.4	8.9	10.3	9.6	9.2	11.2*
Married	%	43.8	43.5	54.8	51.8	52.2	53.0	53.4	53.6	51.7**
Eating green vegetables almost daily	%	24.4	33.3	30.9	33.0	32.1	32.2	31.6	30.6	34.4
No prior disease history (cancer, MI, or stroke)	%	61.2	61.4	63.1	64.1	65.4	64.1	66.3	65.4	55.9
Number		242	345	1,062	1,832	5,596	3,107	2,234	768	331

MI, myocardial infarction.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Mantel–Haenszel test adjusting for age categories.

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without a disease history of cancer, myocardial infarction and/or stroke were analyzed because these factors were known to influence both BMI and mortality (11–14). In addition to age-adjusted HRs, we calculated HRs adjusting for the following potential confounding factors: smoking (current smoker, exsmoker, nonsmoker, or unknown), alcohol consumption (current drinker, exdrinker, nondrinker, or unknown), sleep duration per night (<6.4 h, 6.5–8.4 h, ≥8.5 h, or unknown), physical activity (engaging in physical exercise ≥1 h per week and/or walking >1 h/day, others or unknown), education (attended school up to 15 years of age, 18 years, >18 years or unknown), perceived stress (yes, no, or unknown), marital status (married, single, or unknown), frequency of green vegetables consumed (almost daily, not daily, or unknown), and history of cancer, myocardial infarction or stroke (yes, no, or unknown). Those potential confounding factors were queried in a self-administered questionnaire, and the results of validation studies on the physical activity and food frequency questionnaire were reported previously (15,16). Moreover, additional analyses were conducted to exclude those subjects whose events occurred within 3 years after baseline to avoid reverse-causality bias.

We used the SAS program version 9.1 (SAS Institute, Cary, NC) for analyses conducted at the Aichi Medical University Computation Center.

RESULTS

Mean value of BMI was 21.9 among men and 22.5 among women. Proportions of those underweight (BMI: <18.5), overweight (BMI: 25.0–29.9) and obese (BMI: ≥30.0) were 10.9, 12.6, and 0.7% among men, and 10.6, 19.3, and 2.1% among women at baseline, respectively. Compared to those with normal-range BMI, both underweight and overweight/obese men and women were less likely to be drinkers, to sleep for the normal duration, and to be physically active, while they were more likely to suffer from high levels of mental stress (Table 1). Underweight and overweight/obese men were less likely to eat green vegetables, and corresponding women were more likely to be current smokers. However, among men, the proportion of current smokers decreased with increasing BMI. Among both men and women, subjects who were young, married, and free from prior disease history (cancer, myocardial infarction, or stroke) increased according to increasing BMI. Highly educated subjects showed different trends by gender, increasing among men and decreasing among women with increasing BMI.

A total of 5,292 (47.1%) and 3,964 (25.5%) deaths occurred prior to 2003 among men and women, respectively. Those who had moved out of the study areas numbered 1,208 (4.5%), and they were more likely to be women and older than those who were successfully followed. The average follow-up period was 11.2 years (10.6 years for men, 11.7 years for women). Deaths from malignant neoplasms (ICD10: C00–C97), diseases of the circulatory system (I00–I99), pneumonia (J12–J18), and senility (R54) accounted for 32.6, 32.0, 10.3, and 1.8% of total deaths among men and 26.4, 39.0, 7.7, and 3.0% among women, respectively (Table 2). The proportion of those who died from malignant neoplasms was highest in the normal-range BMI group, but diminished as the BMI fluctuated above or below normal. Mortality from diseases of the circulatory system

Table 2 Cause of mortality according to BMI categories

	BMI category									
	<16.0	16.0–16.9	17.0–18.4	18.5–19.9	20.0–22.9	23.0–24.9	25.0–27.4	27.5–29.9	≥30.0	Total
Men										
Number at baseline	126	232	871	1,622	4,670	2,217	1,136	279	77	11,230
Number of deaths										
All causes	94	157	500	831	2,149	936	473	115	37	5,292
%	74.6	67.7	57.4	51.2	46.0	42.2	41.6	41.2	48.1	47.1
Malignant neoplasms	13	42	139	252	762	320	151	35	11	1,725
%[a]	13.8	26.8	27.8	30.3	35.5	34.2	31.9	30.4	29.7	32.6
Diseases of the circulatory system	23	31	150	244	681	329	179	44	15	1,696
%[a]	24.5	19.7	30.0	29.4	31.7	35.1	37.8	38.3	40.5	32.0
Pneumonia	18	27	63	98	219	73	33	9	5	545
%[a]	19.1	17.2	12.6	11.8	10.2	7.8	7.0	7.8	13.5	10.3
Senility	3	2	17	16	40	10	6	0	1	95
%[a]	3.2	1.3	3.4	1.9	1.9	1.1	1.3	0.0	2.7	1.8
Women										
Number at baseline	242	345	1,062	1,832	5,596	3,107	2,234	768	331	15,517
Number of deaths										
All causes	132	121	362	536	1,322	690	519	179	103	3,964
%	54.5	35.1	34.1	29.3	23.6	22.2	23.2	23.3	31.1	25.5
Malignant neoplasms	21	26	65	132	359	213	161	45	24	1,046
%[a]	15.9	21.5	18.0	24.6	27.2	30.9	31.0	25.1	23.3	26.4
Diseases of the circulatory system	48	42	151	210	488	272	199	87	48	1,545
%[a]	36.4	34.7	41.7	39.2	36.9	39.4	38.3	48.6	46.6	39.0
Pneumonia	17	17	39	54	91	49	30	7	3	307
%[a]	12.9	14.0	10.8	10.1	6.9	7.1	5.8	3.9	2.9	7.7
Senility	3	4	15	15	50	15	16	2	0	120
%[a]	2.3	3.3	4.1	2.8	3.8	2.2	3.1	1.1	0.0	3.0

[a]Percentage of deaths per all causes.

Table 3 Hazard ratios and 95% CI of all-cause mortality according to BMI among men aged 65–79

	BMI category								
	<16.0	16.0–16.9	17.0–18.4	18.5–19.9	20.0–22.9	23.0–24.9	25.0–27.4	27.5–29.9	≥30.0
Total									
Person-years at risk	967	1,962	8,565	16,699	50,471	24,168	12,720	3,142	842
Number of deaths	94	157	500	831	2,149	936	473	115	37
Age-adjusted HR	1.99	1.74	1.27	1.16	1.00	0.94	0.91	0.87	1.02
Age-adjusted 95% CI	(1.62–2.45)	(1.48–2.05)	(1.16–1.40)	(1.07–1.25)		(0.87–1.01)	(0.83–1.01)	(0.72–1.05)	(0.74–1.42)
Multivariate HR[a]	1.78	1.66	1.16	1.12	1.00	0.94	0.92	0.89	0.93
Multivariate 95% CI[a]	(1.45–2.20)	(1.41–1.96)	(1.06–1.28)	(1.04–1.22)		(0.87–1.02)	(0.83–1.01)	(0.73–1.07)	(0.67–1.29)
Not current smokers									
Person-years at risk	404	1,024	3,529	7,706	26,355	14,217	7,359	2,050	552
Number of deaths	45	70	203	371	1,008	503	260	66	21
Age-adjusted HR	2.54	1.70	1.41	1.22	1.00	0.98	1.01	0.87	1.04
Age-adjusted 95% CI	(1.88–3.43)	(1.33–2.16)	(1.21–1.64)	(1.08–1.37)		(0.88–1.09)	(0.88–1.16)	(0.67–1.11)	(0.67–1.59)
Multivariate HR[a]	2.24	1.72	1.29	1.20	1.00	0.98	1.01	0.82	0.87
Multivariate 95% CI[a]	(1.66–3.03)	(1.35–2.20)	(1.11–1.50)	(1.07–1.36)		(0.88–1.09)	(0.88–1.16)	(0.64–1.05)	(0.56–1.34)
Physically active									
Person-years at risk	480	992	4,241	8,529	25,511	11,590	5,658	1,303	385
Number of deaths	34	69	201	345	934	399	189	39	11
Age-adjusted HR	1.57	1.84	1.22	1.08	1.00	0.98	0.95	0.83	0.69
Age-adjusted 95% CI	(1.12–2.22)	(1.44–2.35)	(1.04–1.42)	(0.95–1.22)		(0.87–1.10)	(0.81–1.11)	(0.60–1.14)	(0.38–1.25)
Multivariate HR[a]	1.46	1.76	1.11	1.04	1.00	1.00	0.98	0.83	0.65
Multivariate 95% CI[a]	(1.03–2.06)	(1.37–2.25)	(0.95–1.29)	(0.92–1.18)		(0.89–1.13)	(0.84–1.15)	(0.60–1.15)	(0.36–1.19)
No history of cancer, MI or stroke									
Person-years at risk	484	1,236	5,231	10,750	33,931	15,849	8,331	2,069	548
Number of deaths	40	91	282	483	1,323	524	289	64	21
Age-adjusted HR	1.78	1.77	1.29	1.15	1.00	0.87	0.93	0.82	1.04
Age-adjusted 95% CI	(1.30–2.44)	(1.43–2.19)	(1.13–1.47)	(1.04–1.28)		(0.79–0.97)	(0.82–1.06)	(0.63–1.05)	(0.68–1.60)
Multivariate HR[a]	1.68	1.80	1.20	1.14	1.00	0.91	0.93	0.84	0.99
Multivariate 95% CI[a]	(1.22–2.30)	(1.45–2.23)	(1.05–1.36)	(1.03–1.26)		(0.82–1.00)	(0.82–1.06)	(0.66–1.09)	(0.64–1.53)
Excluded those who died within 3 years									
Person-years at risk	920	1,209	8,407	16,472	49,949	23,952	12,612	3,104	840
Number of deaths	65	124	406	708	1,869	818	424	96	36
Age-adjusted HR	1.72	1.68	1.21	1.15	1.00	0.94	0.93	0.83	1.15
Age-adjusted 95% CI	(1.34–2.20)	(1.40–2.01)	(1.09–1.35)	(1.05–1.25)		(0.86–1.02)	(0.84–1.04)	(0.67–1.01)	(0.83–1.60)
Multivariate HR[a]	1.56	1.62	1.11	1.11	1.00	0.95	0.94	0.84	1.07
Multivariate 95% CI[a]	(1.22–2.00)	(1.35–1.94)	(0.99–1.23)	(1.02–1.21)		(0.88–1.03)	(0.84–1.04)	(0.69–1.04)	(0.77–1.49)

CI, confidence interval; HR, hazard ratio; MI, myocardial infarction.
[a]Adjusted for smoking, drinking, physical activity, sleep duration, stress, education, marital status, green vegetables, stroke, MI, cancer (includes unknown groups).

seemed to increase as the BMI increased, except for a minor increase in the severely thin group. Mortality from pneumonia showed an obvious inverse association with BMI, and senility was rare among overweight/obese groups.

Tables 3 and 4 showed the HRs by gender of all-cause mortality by BMI categories. Compared with the mid-normal-range group (BMI: 20.0–22.9), multiple-adjusted HRs of all-cause mortality for underweight groups were statistically higher among both men and women, with the highest mortality risk found in the severely thin group (BMI: <16.0) as 1.78 (95% confidence interval: 1.45–2.20) in men, and 2.55 (2.13–3.05) in women. Even within the normal-range group, the lower normal range (BMI: 18.5–19.9) showed a statistically elevated risk compared with the mid normal range (HR: 1.12 in men and 1.22 in women). In contrast, overweight subjects showed no relation with risk elevation among either men or women.

Table 4 Hazard ratios and 95% CI of all-cause mortality according to BMI among women aged 65–79

	BMI category								
	<16.0	16.0–16.9	17.0–18.4	18.5–19.9	20.0–22.9	23.0–24.9	25.0–27.4	27.5–29.9	≥30.0
Total									
Person-years at risk	2,301	3,729	11,814	20,849	65,923	37,144	26,483	9,218	3,844
Number of deaths	132	121	362	536	1,322	690	519	179	103
Age-adjusted HR	2.66	1.52	1.45	1.23	1.00	0.98	1.06	1.07	1.37
Age-adjusted 95% CI	(2.22–3.18)	(1.26–1.83)	(1.29–1.63)	(1.11–1.36)		(0.90–1.08)	(0.96–1.17)	(0.91–1.25)	(1.12–1.68)
Multivariate HR[a]	2.55	1.47	1.42	1.22	1.00	0.96	1.01	0.98	1.24
Multivariate 95% CI[a]	(2.13–3.05)	(1.22–1.77)	(1.26–1.59)	(1.11–1.35)		(0.88–1.06)	(0.92–1.12)	(0.84–1.14)	(1.01–1.52)
Not current smokers									
Person-years at risk	1,631	2,678	9,147	16,306	52,259	29,950	21,359	7,410	3,086
Number of deaths	99	88	273	418	1,033	544	406	145	75
Age-adjusted HR	2.89	1.53	1.43	1.23	1.00	0.98	1.06	1.09	1.24
Age-adjusted 95% CI	(2.35–3.55)	(1.23–1.90)	(1.25–1.63)	(1.10–1.38)		(0.89–1.09)	(0.94–1.18)	(0.92–1.30)	(0.98–1.57)
Multivariate HR[a]	2.72	1.48	1.40	1.24	1.00	0.97	1.02	1.00	1.14
Multivariate 95% CI[a]	(2.21–3.35)	(1.19–1.84)	(1.22–1.60)	(1.10–1.39)		(0.87–1.07)	(0.91–1.14)	(0.84–1.19)	(0.90–1.44)
Physically active									
Person-years at risk	726	1,605	5,078	9,141	29,875	16,085	11,503	3,578	1,325
Number of deaths	32	41	126	200	518	253	171	57	30
Age-adjusted HR	2.32	1.45	1.46	1.25	1.00	1.01	1.00	1.10	1.37
Age-adjusted 95% CI	(1.62–3.31)	(1.05–1.99)	(1.21–1.78)	(1.06–1.47)		(0.87–1.17)	(0.84–1.19)	(0.84–1.45)	(0.95–1.99)
Multivariate HR[a]	2.17	1.41	1.42	1.23	1.00	0.99	0.97	1.02	1.37
Multivariate 95% CI[a]	(1.52–3.11)	(1.02–1.94)	(1.17–1.73)	(1.05–1.45)		(0.85–1.15)	(0.82–1.16)	(0.77–1.34)	(0.95–1.98)
No history of cancer, MI or stroke									
Person-years at risk	1,477	2,327	7,659	13,739	43,948	24,071	17,939	6,164	2,233
Number of deaths	77	75	225	327	835	439	322	111	53
Age-adjusted HR	2.53	1.57	1.49	1.22	1.00	1.03	1.05	1.09	1.32
Age-adjusted 95% CI	(2.01–3.20)	(1.24–1.99)	(1.28–1.72)	(1.07–1.38)		(0.92–1.15)	(0.93–1.20)	(0.89–1.33)	(1.00–1.74)
Multivariate HR[a]	2.38	1.52	1.44	1.21	1.00	1.02	1.02	1.01	1.21
Multivariate 95% CI[a]	(1.88–3.01)	(1.20–1.92)	(1.24–1.67)	(1.06–1.37)		(0.90–1.14)	(0.89–1.16)	(0.83–1.23)	(0.92–1.60)
Excluded those who died within 3 years									
Person-years at risk	2,269	3,697	11,722	20,709	65,577	36,995	26,351	9,164	3,825
Number of deaths	111	101	319	465	1,177	626	464	157	94
Age-adjusted HR	2.63	1.45	1.45	1.20	1.00	1.00	1.06	1.05	1.41
Age-adjusted 95% CI	(2.16–3.19)	(1.18–1.77)	(1.28–1.64)	(1.08–1.34)		(0.91–1.10)	(0.95–1.18)	(0.89–1.24)	(1.14–1.74)
Multivariate HR[a]	2.52	1.40	1.42	1.20	1.00	0.98	1.02	0.97	1.28
Multivariate 95% CI[a]	(2.07–3.06)	(1.14–1.72)	(1.25–1.61)	(1.08–1.34)		(0.89–1.08)	(0.92–1.14)	(0.82–1.15)	(1.04–1.59)

CI, confidence interval; HR, hazard ratio; MI, myocardial infarction.
[a]Adjusted for smoking, drinking, physical activity, sleep duration, stress, education, marital status, green vegetables, stroke, MI, cancer (includes unknown groups).

In addition, obesity (BMI: ≥30.0) did not elevate the all-cause mortality risk among men, though a slight statistically significant risk was observed among women (HR: 1.24) compared with the mid-normal-range group. Subcohort analyses of noncurrent smokers, physically active subjects, and those without major disease at baseline did not alter the risk estimation dramatically. Excluding events occurring within 3 years also produced no change in the effects on all-cause mortality of the underweight and overweight/obese groups.

DISCUSSION

Using a dataset of a large population-based cohort study of older Japanese subjects aged 65–79 who were followed for >10 years on average, we found that a BMI between 20.0 and 29.9 was associated with a minimum risk of all-cause mortality. This wide range was unchanged when our analysis was limited to subjects who could be followed for at least 3 years from baseline. Moreover, the results were essentially unchanged when subcohort analyses were conducted of those who were not currently smoking, were physically active, or were without a history of cancer, cardiovascular disease, or stroke.

The key advantages of our study were its large-scale cohort with subjects from all over Japan, a long follow-up period of >10 years, and adjustments for known confounders. These advantages allowed us to adopt narrow categories of BMI to examine the association with all-cause mortality among older adults. Moreover, subcohort analyses could be performed considering several factors which influence both body composition and all-cause mortality, especially among the older adults, such as (i) heavy and lengthy periods of smoking (14,17), (ii) physical activity (11), and (iii) subclinical diseases (12).

Risk elevation among thin older adults with results similar to ours was reported by many other cohort studies (1,18,19). There may be several explanations for this association so commonly observed among older adults. First, because lean mass acts as a nutritional preserve (4), and aging itself results in a decline in immune response, such thin older adults may be less resistant to infection (20). Actually, deaths from pneumonia were more prevalent among underweight subjects compared with normal or overweight subjects in our cohort. Second, preexisting disease may be linked to both thinness and an increased risk of death. As shown in Table 1, there were more older adults among low-BMI subjects compared with those in other groups, suggesting that age-related diseases cause weight loss. However, excluding the first 3 years of follow-up did not alter that result. Though the purpose of this article was to examine the association between BMI and all-cause mortality, further investigations into the effects of BMI on cause-specific mortality may help us to better understand the relationship of BMI to lean and/or fat mass, and susceptibility to death among older adults. Third, a confounding influence of smoking may exist, because smokers tend to lose weight more readily than nonsmokers (21), and smoking is known to reduce life expectancy (22). Even if such a confounding effect should exist, subcohort analysis of noncurrent smokers revealed that thin subjects who did not smoke also had a higher risk of all-cause mortality, which suggests that a confounding effect from smoking is not the main explanation. Nevertheless, we cannot rule out the possibility that, even with a careful determination of known confounding variables in the present analysis, other undetected factors related to increased mortality risk among thin older adults might have confounded the association between BMI and mortality.

Overweight/obesity is related to excess mortality among both younger and middle-aged populations (1,23,24), and the cut-off points recommended by World Health Organization (3) are mainly based on them. Though some studies have found that the risk of death among older adults was associated with obesity/overweight (2,12,18), the meta-analysis by Janssen and Mark showed no risk elevation for overweight subjects (estimated risk 1.00 with 95% confidence interval: 0.97–1.03), and a significant though very small risk elevation for obese subjects (1.10, 1.06–1.13) (4). Our study showed no increased risk elevation in overweight/obese subjects (except in obese (BMI: ≥30.0) women), and our results were not altered even among some subcohorts. Although the reason for these inconsistent findings is unclear, explanations of why the weak or absent effect of overweight/obesity on all-cause mortality was observed among the older adults in our study may include the following. First, some individuals who were susceptible to the adverse effects of a high BMI may have already died in youth or middle-age, whereas the older adults with a high BMI who survived may have developed a resistance to the effect of overweight/obesity (4,25). Because obesity in women was found to be associated with increased mortality, it is also possible that severely obese men might have been underrepresented in the present sample (self-selection). Second, the possible protective effects of being overweight reflected by a high BMI (such as nutritional reserve) may have prevailed over its negative effects on all-cause mortality in the elderly population (4). Third, a recent study has shown that the prevalence of a clustering of cardiometabolic risk factors among normal-weight individuals was higher in older age groups compared with that in young and middle-aged subjects (26). Thus, the elevated risk of mortality in the normal-weight group among older adults may have caused a relative risk reduction in the overweight/obese groups. As a result, the BMI in older adults may not be a reliable predictor of mortality risk, especially that from cardiovascular diseases, because the variability of BMI in this age group does not adequately reflect that of other intermediate variables leading to disease.

There are some study limitations we should discuss. First, our data were based on self-reported rather than measured heights and weights. Spencer *et al.* compared self-reported and measured height, weight, and BMI among subjects aged 35–76 years. They found that height was overestimated and weight was underestimated, resulting in underestimation of BMI, especially among heavier men and women (27). Thus, we could not exclude the possibility that overweight/obese older adults underestimated their BMI more often than those with a normal BMI, and consequently, misclassifications leading to an underestimation of overweight/obese risk may have

occurred. However, according to the same authors (27), normal BMI category men and women were the least likely to be incorrectly allocated to another BMI category, and underweight participants were also less likely to be misclassified into the normal range than overweight/obese subjects, making it somewhat unlikely that overestimations of underweight risk might occur. Second, we have no information on body fat or its distribution, such as the ratio of waist-to-hip circumferences. Both high-body fat and low fat-free mass are known to be independent predictors of overall mortality (28). Moreover, Simpson *et al.* reported that, among women, central adiposity was a better predictor of mortality than BMI (29). A large-scale cohort study among older adults that includes such information will be required to investigate the relationship between body composition and mortality. Finally, it should be kept in mind that we did not examine any relationships between weight history and mortality. Moreover, a review by Bales and Buhr revealed the benefits of maintaining weight in older persons who become obese after age 65 (30). Therefore, the result of our observational study should not be used to dismiss the necessity of weight reduction among all obese older adults. In addition, we do not recommend that underweight older adults should gain weight based on our results, because ours was not an interventional study.

In conclusion, we found an elevated risk of all-cause mortality among thin Japanese older adults and a wide range of BMI between 20.0 and 29.9 that showed the lowest mortality risk to be among both older men and women.

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DISCLOSURE
The authors declared no conflict of interest.

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CHEMICAL CONFIRMATION OF THE STRUCTURE OF A MUTAGENIC AMINOPHENYLNORHARMAN, 9-(4'-AMINOPHENYL)-9*H*-PYRIDO[3,4-*b*]INDOLE : AN AUTHENTIC SYNTHESIS OF 9-(4'-NITROPHENYL)-9*H*- PYRIDO[3,4-*b*]INDOLE AS ITS RELAY COMPOUND

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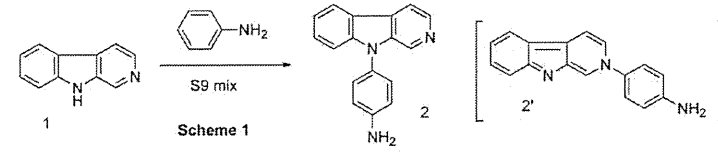
Abstract – 9-(4'-Aminophenyl)-9*H*-pyrido[3,4-*b*]indole **2** is a mutagenic compound produced by non-mutagenic norharman **1** and aniline in the presence of S9 mix. 9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole **4**, the relay compound for synthesis of **2**, was synthesized starting from ethyl indole-2-aldehyde **12** via initial *N*-(4-nitro)phenylation of the indole nucleus, elongation of the 2-aldehyde substituent, and then construction of the pyridine nucleus in order to ensure the nitrogen substitution in **2**.

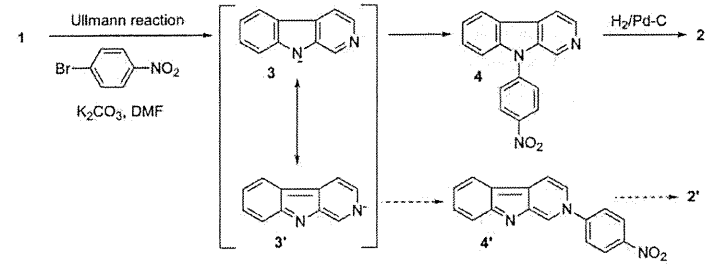
INTRODUCTION

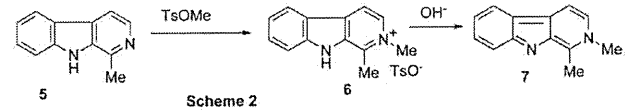
Sugimura et al.[1] reported that norharman **1** (9*H*-pyrido[3,4-*b*]indole, β-carboline) itself is not mutagenic to *Salmonella* strains, but becomes mutagenic to *S. typhimurium* TA98 and YG1024 with S9 mix in the presence of non-mutagenic aromatic amines such as aniline and *o*-toluidine. In a



Scheme 1

subsequent report[2] they isolated mutagenic compound **2** produced by the reaction between norharman and aniline with S9 mix (Scheme 1). In order to elucidate the structure, one of the potential structures, 9-(4'-aminophenyl)-9*H*-pyrido[3,4-b]indole **2**, was synthesized[2] via Ullmann reaction of norharman **1** with 4-bromonitrobenzene, followed by catalytic hydrogenation. The synthetic sample was identical to the natural one and the spectral data of the product supported the structure of **2** but not **2'**. The synthetic strategy was based on the fact that Ullmann reaction of indoles with aryl halides proceeded at its NH position.[3] However, if the reaction occurs on the pyridine nitrogen of **1** via its basicity or another resonance structure **3'**, the product should be compound **4'** (Scheme 1 and 2), whose structure would be much more unstable than the structure **4**, as it has neither benzene, indole, nor pyridine aromaticity any longer. Thus, such a compound is thought to be difficult to produce. On the other hand, it was recently reported[4] that N_A-methylammonium harman **6** derived from harman **5** was basified to yield the compound **7**, whose skeleton is the same as those of **2'** and **4'** (Scheme 2). In this paper we report the authentic synthesis of the relay compound **4** in order to ensure the nitrogen substitution of the substituted phenyl group in **2**.



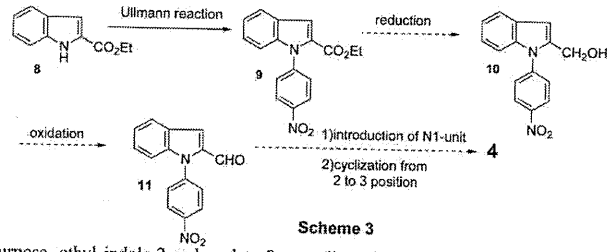


Scheme 2

RESULTS AND DISCUSSION

The synthetic strategy for the synthesis of the relay compound **4** was designed as shown in Scheme 3. The key point is the initial (4-nitro)phenylation at the 1-nitrogen position in the indole nucleus, followed by elongation of the 2-substituent and cyclization to form the pyridine nucleus.

The usual construction of the pyridine ring in the indole nucleus for synthesis of the 9*H*-pyrido[3,4-*b*]indole nucleus is cyclization of the 3-substituent of the tryptamine derivative to the 2-position of the indole nucleus as seen in the Bischler-Napieralski reaction, Pictet-Spengler reaction and so on. On the other hand, there are few methods for cyclization of the 2-substituent to the 3-position of the indole nucleus. Several years ago we developed a method for 9*H*-pyrido[3,4-*b*]indole synthesis of the latter type in the course of the synthetic study of 4-oxo-β-carboline.[5] We applied this method in the present strategy.

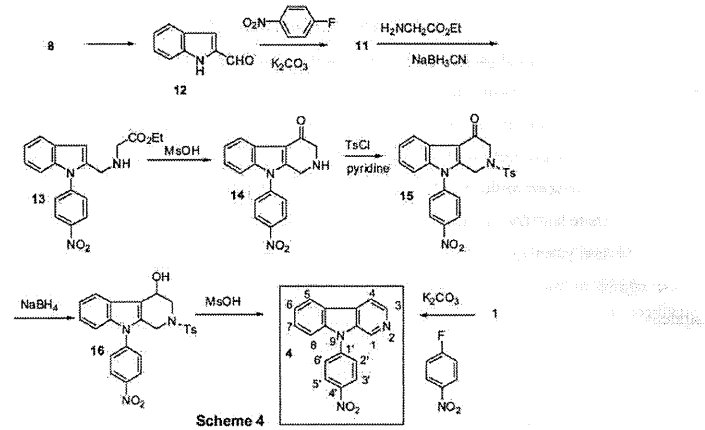


Scheme 3

For this purpose, ethyl indole-2-carboxylate **8** was allowed to react with 4-fluoronitrobenzene to give ethyl *N*-(4'-nitrophenyl)indole-2-carboxylate **9**. However, the reduction of ester carbonyl of **9** with $LiAlH_4$ was not successful (Scheme 3). Thus, the reaction scheme to prepare the aldehyde **11** had to be changed. The synthetic route was changed as in Scheme 4.

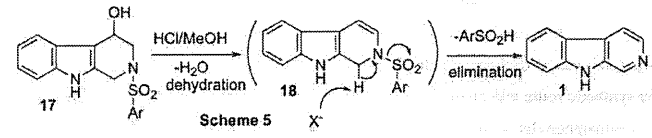
The first *N*-(4-nitro)phenylation of indole-2-carboxaldehyde[5] **12** prepared from **8**, which we feared to proceed with difficulty due to the sensitive reactivity of aldehyde functionality, went much better than expected (51% yield). The *N*-(4'-nitrophenyl)indole-2-carboxaldehyde **11** thus obtained was allowed to react with ethyl aminoacetate and then sodium cyanoborohydride to give the *N*-indolic aminoacetate **13**. The cyclization of **13** with methanesulfonic acid gave the cyclized amino ketone **14**. The aminoketone **14** was treated with tosyl chloride in the presence of pyridine to give the corresponding tosylamide **15** in good yield. The subsequent process of cyclic amino ketone resembling **14** to the target 9*H*-pyrido[3,4-*b*]indole nucleus has already been developed.[6]

The reduction of the ketone of **15** to the hydroxyl group with a large excess amount of sodium borohydride proceeded to give the alcohol **16** in good yield. The last and important dehydration and aromatization processes (two successive β-eliminations) were examined for the present reaction.



Scheme 4

The reaction[6] involved dehydration of the alcohol and β-elimination process around the sulfonyl group with HCl in MeOH, as shown in Scheme 5. In the present case the reaction did not proceed well with HCl in MeOH in several trials, probably due to its insolubility.



Scheme 5

After several acidic conditions were tried, the alcohol **16** was allowed to react with methanesulfonic acid. The target compound **4** was finally obtained from the basic layer in this reaction (20% yield). The product was identified with the already[2] and freshly synthesized sample directly via the Ullmann reaction from **1** and 4-bromo-(or 4-fluoro)nitrobenzene in the presence of K_2CO_3. It was proved that the Ullmann reaction of **1** proceeded at the indolic NH position even on the 9*H*-pyrido[3,4-*b*]indole nucleus. It is worth noting that the Ullmann reaction of **1** with 4-fluoronitrobenzene without K_2CO_3 did not proceed at all. This means that the formation of nitrogen anion is necessary for Ullmann reaction of indole and pyridine nitrogen cannot take Ullmann reaction directly. Thus, the structure **2** was chemically determined. Using this scheme, it may be possible to develop a new strategy for 9*H*-pyrido[3,4-*b*]indole synthesis that involves cyclication of the 2-substituent toward the 3-position of the indole skeleton.

EXPERIMENTAL

All melting points were measured on a hot stage micro-melting points apparatus (Yanagimoto) and are uncorrected. Elemental analyses were conducted with a Yanaco CHN CORDER MT-6. The ^{1}H-NMR spectra were measured with a Bruker Ultrashield^{TM} 400 Plus (400MHz) spectrometer. Deuteriochloroform was used as the solvent with tetramethylsilane as an internal reference. MS spectra were measured on JEOL JMS-GC-mate II and JEOL JMS-600H spectrometers. IR spectra were recorded on a Shimadzu FTIR-8400S spectrometer. For column chromatography, Silica gel 60 (70-230 mesh ASTM; Merck) was used.

1-(4'-Nitrophenyl)indole-2-carboxaldehyde 11

A solution of indole-2-carboxaldehyde[5] **12** (1.80 g, 12.4 mmol), 4-fluoronitrobenzene (5.19 g, 36.8 mmol), and powdered anhydrous K_2CO_3 (5.14 g, 37.2 mmol) in anhydrous DMF (27 mL) was heated with stirring at 100 °C for 1.5 h. The reaction mixture was poured onto water (150 mL), and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The residue (6.38 g) was chromatographed over SiO_2 with toluene as eluent to give the target compound **11** (1.70 g, 51%). Recrystallization of a part of the compound from a mixture of AcOEt and hexane gave pale yellow columns, mp 170-172 °C. *Anal.* Calcd for $C_{15}H_{10}N_2O_3$: C, 67.67; H, 3.79; N, 10.52. Found: C, 67.98; H, 3.95; N, 10.43. MS ($C_{15}H_{10}N_2O_3$): *m/z*266 (M^+). IR ν max(KBr)cm^{-1}: 1683 (sh), 1672 (CO). ^{1}H-NMR ($CDCl_3$) δ : 7.23-7.58 (6H, m, $C_{3,5,6,7,2',6'}$-H), 7.83 (2H, m, C_4-H), 8.41 (2H, d, *J*=8.0 Hz, $C_{3'}$,$C_{5'}$-H), 9.89 (1H, s, CHO).

Ethyl [1-(4'-Nitrophenyl)indole-2-ylmethyl]aminoacetate 13

To a muddy solution of 1-(4'-nitrophenyl)indole-2-carboxaldehyde **11** (724 mg, 2.77 mmol) and ethyl aminoacetate hydrochloride (1.12 g, 8.16 mmol) in ethanol (30 mL) was added triethylamine (1.17 mL, 8.16 mmol) and $NaBH_3CN$ (685 mg, 10.9 mmol) successively with stirring under ice-cooling. The reaction mixture (muddy state) was stirred under ice-cooling for 15 min and then at rt for an additional 3 h. Then, the reaction mixture was poured onto water and extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo* to dryness to give a pale yellow residue. The crude products were chromatographed over SiO_2. Elution with toluene, followed by toluene-AcOEt (10:1), gave the target compound **13** (713 mg, 74%) as a pale yellow oil. MS ($C_{19}H_{19}N_3O_4$): *m/z* 353 (M^+). HRMS: Calcd for $C_{19}H_{19}N_3O_4$, 353.1376; Found, 353.1378. IR ν max($CHCl_3$)cm^{-1}: 3684, 3620 (NH), 1734 (C=O). ^{1}H-NMR δ :1.23 (3H, t, *J*=8.0 Hz, -$CH_2\underline{CH_3}$), 3.41 (2H, s, -CH_2NH-), 3.94 (2H, s, -NCH_2CO-), 4.12 (2H, *J*=8.0 Hz, -$OC\underline{H_2}CH_3$), 6.75 (1H, s, C_3-H), 7.18-7.26 (3H, m, $C_{5-6,7}$-H), 7.63 (1H, m, C_4-H), 7.73 (2H, *J*=8.0 Hz, $C_{2',6'}$-H), 8.41 (2H, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-one 14

A mixture of ethyl [1-(4'-nitrophenyl)indole-2-ylmethyl]aminoacetate **13** (513 mg, 1.45 mmol) and methanesulfonic acid (7 mL) was stirred at 45 °C for 45 min, and then 70 °C for 1 h. The reaction mixture was poured onto water (50 mL), basified with K_2CO_3, and extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave a solid (425 mg). This solid was chromatographed over SiO_2 (12 g). Elution with $CHCl_3$, followed by AcOEt, gave a small amount of the starting material and unknown compounds. Further elution with a mixture of AcOEt and EtOH (10:1) gave the target compound **14** (298 mg, 67%). A part of the sample was recrystallized from acetone to give pale yellow fine needles, mp 215-217 °C (decomp). MS ($C_{17}H_{13}N_3O_3$): *m/z*307 (M^+, 25% of base peak), 252(base peak). HRMS: Calcd for $C_{17}H_{13}N_3O_3$, 307.0957; Found,307.0964. IR ν max(KBr)cm^{-1}:3326(NH), 1649(CO). ^{1}H-NMR δ : 2.23 (1H, br.s, NH), 3.67 (2H, s, C_3-H), 4.13 (2H, s, C_1-H), 7.21-7.45 (3H, m, $C_{6,7,8}$-H), 7.62 (2H, d, *J*=8.0 Hz, $C_{2',6'}$-H), 8.30 (1H, m, C_5-H), 8.50 (2H, d, *J*=8.0 Hz, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-2-tosyl-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-one 15

To a suspension of 9-(4'-nitrophenyl)-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole **14** (265 mg, 0.862 mmol) in pyridine (7 mL) was added TsCl (493 mg, 2.59 mmol) under ice-cooling. The mixture was stirred under ice-cooling for 15 min and at rt for an additional 1 h. The reaction mixture was poured onto water, extracted with $CHCl_3$, washed with dil. HCl aq. and water, and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the target compound **15** (349 mg, 88%). A part of the compound was recrystallized from a mixture of DMF and EtOH to give almost colorless very fine needles, mp 253-258 °C (decomp). *Anal.* Calcd for $C_{24}H_{19}N_3O_5S$: C, 62.46; H, 4.15; N, 9.11: Found: C, 62.42, H; 4.18, N; 8.58. MS: *m/z* 461 (M^+,15% of base peak), 306 (base peak). HRMS:Calcd for $C_{24}H_{19}N_3O_5S$, 461.1045; Found,461.1043. IR ν max(KBr)cm^{-1}:1664(CO). ^{1}H-NMR δ : 2.50 (3H, s, arom-CH_3), 4.11 (2H, s, C_3-H), 4.76 (2H, s, C_1-H), 7.20-7.45 (7H, m, arom-H), 7.87 (1H, dd, *J*=8.0 and 2.0 Hz, C_5-H), 7.92 (2H, d, *J*=8.0 Hz, Ts-ortho-H), 8.56 (2H, d, *J*=8.0 Hz, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-2-tosyl-2,3,4,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-ol 16

The tosyl ketone **15** (33 mg, 0.0715 mmol) was added to a mixture of $CHCl_3$ (1.5 mL) and MeOH (4 mL). To the resulting suspension was added $NaBH_4$ (270 mg, 7.15 mmol) under ice-cooling to prevent generation of heat at the beginning and then the whole was stirred for 4.5 h at rt. The reaction mixture was poured onto water and extracted with $CHCl_3$. The organic layer was washed with brine and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the target alcohol **16** as yellowish powder (31 mg, 94%). This sample showed one spot on TLC (SiO_2, toluene-AcOEt = 2:1), and was

used for the next reaction. A part of the powder was recrystallized from acetone-MeOH to give pale yellow powder, mp 175-177 °C (decomp). *Anal.* Calcd for $C_{24}H_{21}N_3O_3S$: C, 62.19; H, 4.57; N, 9.07. Found: C, 61.83; H, 4.68; N, 8.85. MS: 463 (M^+, 5.9% of the base peak), 252 (base peak). HRMS; Calcd, 463.1202; Found, 463.1208. IR ν max(KBr)cm^{-1}: 3482(OH). ^{1}H-NMR δ : 2.44 (3H, s, arom-CH_3), 3.16,3.96,4.55 (4H, aliph-H), 5.12 (1H, br. d, *J*=12.0 Hz, C_4-H), 7.24-7.78 (10H, m, arom-H), 8.46 (2H, d, *J*=8.0 Hz, $C_{3'}$, $C_{5'}$-H).

9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole 4 from 9-(4'-nitrophenyl)-2-(toluene-4"-sulfonyl)-2,3,4,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-ol 16

The above-mentioned alcohol **16** (40 mg, 0.086 mmol) was dissolved in methanesulfonic acid (3 mL) and stirred for 4 h at rt. The reaction mixture was poured onto water and extracted out with AcOEt. The aqueous layer was basified with K_2CO_3 and extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the crude product. The crude product was purified with column-chromatography [SiO_2 (8 g), $CHCl_3$] to give yellow powder (5 mg, 20%), mp 188-190 °C. This sample was identified with the relay compound[2] derived from Ullmann reaction of norharman **1** and 4-bromonitrobenzene (or 4-fluoronitrobenzene) as described below, based on their NMR spectra and TLC behavior. ^{1}H-NMR δ : 7.44 (1H, m, C_6-H), 7.56-7.62 (2H, m, $C_{7,8}$-H), 7.85 (2H, d, *J*=8 Hz, $C_{2',6'}$-H), 8.05 (1H, d, *J*=4.0 Hz, C_4-H), 8.23 (1H,d, *J*=8.0 Hz, C_5-H), 8.53 (2H, d, *J*=8.0 Hz, $C_{3',5'}$-H), 8.59 (1H, br. d, *J*=4.0 Hz, C_3=H), 8.95(1H, s, C_1-H).

9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole 4 *via* Ullmann reaction[2] from norharman 1

A mixture of norharman **1** (40 mg, 0.238 mmol), 4-fluoronitrobenzene (66 mg, 0.476 mmol) and powdered anhydrous K_2CO_3 (99 mg, 714 mmol) was added to DMF (3 mL) and the whole was heated at 100 °C with stirring for 3 h. The reaction mixture was poured onto water (60 mL) and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The resulting mass was purified over column chromatography (SiO_2, $CHCl_3$) to give the target compound (79 mg, quantitative). This sample was recrystallized from $CHCl_3$-MeOH and then treated with $CHCl_3$ to give pale yellow needles, mp 192-192.5 °C. The sample obtained from recrystallization from $CHCl_3$-MeOH contained MeOH in its crystals. The crystals were dried at 100 °C *in vacuo* over night for elemental analysis. *Anal.* Calcd for $C_{17}H_{11}N_3O_2$: C; 70.58, H; 3.83, N; 14.53. Found: C; 70.60, H; 3.97, N; 14.53.

Ethyl 1-(4'-nitrophenyl)indole-2-carboxylate 9

In anhydrous DMF (3 mL) was added ethyl indole-2-carboxylate (299 mg, 1.58 mmol), 4-fluoronitrobenzene (417 mg, 3 mmol), and powdered anhydrous K_2CO_3 (304 mg, 2.2 mmol). The whole was heated at 100 °C under stirring for 14.5 h. The reaction mixture was poured onto water, and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (702 mg) was chromatographed over SiO_2 and eluted with toluene to give the target compound **9** (266 mg, 54%). A part of this compound was recrystallized from AcOEt-hexane to give pale yellow plates, mp 133-135 °C. *Anal.* Calcd for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.55; N, 9.03. Found: C, 65.99; H, 4.64; N, 8.76. MS ($C_{17}H_{14}N_2O_4$): 310 (M^+). IR ν max(KBr)cm^{-1}: no NH, 1704 (CO). ^{1}H-NMR δ : 1.29 (3H, t, *J*=8.0 Hz, -$CH_2\underline{CH_3}$), 4.25 (2H, t, *J*=8.0 Hz, -$O\underline{CH_2}CH_3$), 7.13 (1H, d, *J*=1.5 Hz, C_3-H), 7.23-7.36 (3H, m, indolic Hs), 7.52-7.56 (3H, m, $C_{2'}$,$C_{6'}$,an indolic H), 7.76 (1H, d, *J*=9.0 Hz, C_4-H), 8.40 (2H, d, *J*=9.0 Hz, $C_{3'}$,$C_{5'}$-H).

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Low-dose carcinogenicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline in rats: Evidence for the existence of no-effect levels and a mechanism involving p21$^{Cip/WAF1}$

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The carcinogenicity of the low amounts of genotoxic carcinogens present in food is of pressing concern. The purpose of the present study was to determine the carcinogenicity of low doses of the dietary genotoxic carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and to investigate mechanisms by which IQ exerts its carcinogenic effects. A total of 1595 male F344 rats were divided into seven groups and administered with IQ at doses of 0, 0.001, 0.01, 0.1, 1, 10 and 100 p.p.m. in the diet for 16 weeks. We found that IQ doses of 1 p.p.m. and below did not induce preneoplastic lesions in either the liver or the colon, while IQ doses of 10 and 100 p.p.m. induced preneoplastic lesions in both of these organs. These results demonstrate the presence of no-effect levels of IQ for both liver and colon carcinogenicity in rats. The finding that p21$^{Cip/WAF1}$ was significantly induced in the liver at doses well below those required for IQ mediated carcinogenic effects suggests that induction of p21$^{Cip/WAF1}$ is one of the mechanisms responsible for the observed no-effect of low doses of IQ. Furthermore, IQ administration caused significant induction of CYP1A2 at doses of 0.01–10 p.p.m., but administration of 100 p.p.m. IQ induced CYP1A1 rather than CYP1A2. This result indicates the importance of dosage when interpreting data on the carcinogenicity and metabolic activation of IQ. Overall, our results suggest the existence of no-effect levels for the carcinogenicity of this genotoxic compound. (*Cancer Sci* 2011; 102: 88–94)

Exposure to environmental carcinogens is one of the most significant causes of human cancers. Determination of the dose-response relationship between carcinogen exposure and induction of cancer is one of the most important areas of chemical risk assessment. Of particularly high priority is the cancer risk assessment of dietary carcinogens.

Heterocyclic amines (HCA) are well known dietary genotoxic carcinogens derived from cooked protein-rich foods such as meat and fish,[1–3] and the carcinogenicities of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) have been widely investigated in various animal models. MeIQx induces cancers of the liver, zymbal gland, skin and clitoral gland in rats,[4] and caners of the liver and lung, and lymphoma and leukemia in mice.[5] PhIP induces colon cancers and mammary gland cancers in rats,[6] and lymphomas in mice.[7] IQ induces cancers of the liver, colon, mammary and zymbal glands in rats, caners of the liver, lung and forestomach in mice, and cancer of the liver in non-human primates.[8–10] MeIQx and PhIP are classified as category 2B compounds (possibly carcinogenic to humans) and IQ is classified as a category 2A compound (probably carcinogenic to humans) by the International Agency for Research on Cancer.[11] Therefore, although the concentrations of HCA in food are low, they constitute a potential hazard, and there is concern regarding the carcinogenic effects of low doses of these HCA.

Based on the view that even minute doses of a genotoxic carcinogen has the potential to produce irreversible deleterious genetic changes in the DNA of a target organ cell and the argument that if sufficient numbers of test animals are used the carcinogenic effect of a minute dose can be demonstrated, it is generally assumed that genotoxic carcinogens exert a non-threshold carcinogenic effect. However, the carcinogenicities of most genotoxic carcinogens are determined by experimental animal carcinogenicity studies using doses that are generally orders of magnitude higher than actual human exposure levels and the dose-response curves obtained are then extrapolated to zero using a non-threshold mathematical model. This approach, however, is being challenged as advancements in the understanding of the molecular mechanisms of carcinogenesis are being made and experimental evidence showing that genotoxic carcinogens do not exert mutagenic and carcinogenic effects at low doses accumulates.[12–19]

Previously, we demonstrated the existence of no-effect levels of MeIQx for both hepatocarcinogenicity and *in vivo* mutagenicity in various carcinogenesis models in different rat strains.[17,20–22] It has also been shown that low doses of PhIP do not exert either initiation or promotion activities in colon carcinogenesis in the rat.[23,24] However, little is known about the carcinogenic potential of low doses of IQ.

In addition, little is known about the mechanisms underlying the carcinogenicities of lower doses of HCA, but incorporation of mechanistic information is critical for quantitative cancer risk assessment. The purpose of the present study is to determine the relationship between administration of low doses of IQ and induction of preneoplastic lesions in the liver and colon in rats, and to investigate carcinogenic mechanisms of action of various doses of IQ by evaluating DNA-adduct formation, oxidative DNA damage and expression levels of genes involved in metabolic activation of IQ, cell proliferation and DNA damage repair in the liver.

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Materials and Methods

Chemical and diets. IQ was purchased from Nard Institute Ltd (Osaka, Japan) with a purity of 99.9%. Basal diets (powdered MF; Oriental Yeast Co., Tokyo, Japan) and the diets containing IQ were prepared once a month by Oriental Yeast Co.

Animals. A total of 1595 male F344 rats were supplied by Charles River Japan, Inc. (Hino, Shiga, Japan) and were used at 21 days of age. Animals were housed in polycarbonate cages (five per cage) in experimental animal rooms with a targeted temperature of 22 ± 3°C, relative humidity of 55 ± 5% and a 12-h light/dark cycle. Diet and tap water were available *ad libitum* throughout the study.

Experimental design. The animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School. Rats were randomized into seven groups, 245 rats in each of groups 1–6 and 125 rats in group 7. Since the levels of IQ in cooked foods are lower than those of MeIQx and PhIP,[11] IQ dosage and treatment duration in this study were the same as the previous low dose carcinogenicity studies with MeIQx and PhIP.[18,24] Animals were fed diets containing IQ as follows: 0 (group 1, control), 0.001 (group 2), 0.01 (group 3), 0.1 (group 4), 1 (group 5), 10 (group 6) and 100 p.p.m. (group 7) for 16 weeks. Fresh diet was supplied to the animals twice weekly. Bodyweights, food consumption and water intake were measured weekly.

Five rats in each group were killed at week 4 under ether anesthesia. At death, livers were snap frozen in liquid nitrogen and stored at −80°C for examination of IQ-DNA adducts and 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation in the DNA. The remaining rats were killed at the end of week 16 under ether anesthesia for examination of the development of glutathione *S*-transferase placental form (GST-P) positive foci, which is a well-established preneoplastic lesion in the rat liver,[25,26] and aberrant crypt foci (ACF), which is a surrogate marker for preneoplastic lesions in the rat colon.[24,27,28] At death, livers were excised, weighed and then three slices each from the left lateral, medial and right lateral lobes were cut and placed in 10% phosphate-buffered formalin. The remaining liver tissues were snap frozen in liquid nitrogen and stored at −80°C for mRNA expression analysis. Following fixation, liver tissues were embedded in paraffin and processed for histopathological examination.

Examination of GST-P positive foci in the liver. Anti-rat GST-P polyclonal antibody (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan) at a dilution of 1:1000 was used for immunohistochemical staining of GST-P. The GST-P-positive hepatocellular foci composed of two or more cells were counted under a light microscope.[17,18,20,22] Total areas of livers were measured using a color image processor IPAP (Sumica Technos, Osaka, Japan) and the number of GST-P-positive foci per square centimeter of liver tissue was calculated.

IQ-DNA adduct and 8-OHdG formation in livers. IQ-DNA adducts were measured by the ^{32}P-postlabeling method as described previously.[29,30] Levels of 8-OHdG formation in liver DNA were determined by high-performance liquid chromatography with electrochemical detection as previously described.[31]

TaqMan real-time quantitative PCR. The mRNA expression levels of genes involved in IQ metabolism (CYP1A1, CYP1A2 and CYP1B1), DNA damage repair (8-oxoguanine DNA glycosylase [Ogg1], growth arrest and DNA damage-inducible protein 45 [GADD45], AP endonuclease-1 [APE-1], MSH2 and MSH3) and cell cycle regulation (p53 and p21$^{Cip/WAF1}$ and proliferating cell nuclear antigen [PCNA]) were evaluated in the livers by TaqMan real-time quantitative PCR as described previously.[31] Sequence-specific primers and probes (Taqman Gene Expression Assay) were purchased from Applied Biosystems, Inc., Carlsbad, CA, USA. Beta-2-microglobulin (B2M) was used as an internal control.

Examination of ACF in colon. Formation of ACF was examined as described previously.[24] Although ACF consisting of four or more crypts are considered to be better predictors of colon tumor outcome in rats,[32] to ensure that all doses of IQ that have the potential to induce colon carcinogenesis were accounted for, doses of IQ that caused an increase of any size of ACF were considered to have the potential to induce colon carcinogenesis in the present study.[24]

Statistical analysis. All mean values are reported as mean ± SD. Statistical analyses were performed using the Statlight program (Yukms Co., Ltd, Tokyo, Japan). Homogeneity of variance was tested by the Bartlett test. Differences in mean values between the control and IQ-treated groups were evaluated by the 2-tailed Dunnett test when variance was homogeneous and the 2-tailed Steel test when variance was heterogeneous.[22,31] P values <0.05 were considered significant.

Results

General observation. All animals survived to the end of study without any apparent abnormal pathological features. The final average body and liver weights and IQ intake are summarized in Table 1. The final bodyweight of the 100 p.p.m. group was significantly lower than that of the 0 p.p.m. group. Absolute and relative liver weights were significantly decreased in the 0.1 and 1 p.p.m. groups and were significantly increased in the 100 p.p.m. group compared with the 0 p.p.m. group. There were no significant differences in either food or water consumption among groups (data not shown). The intake of IQ was proportional to the administered doses (Table 1). No tumors were found in any organs including the liver and colon in any of the groups.

Induction of GST-P-positive foci in the livers. No histopathological changes were observed in any of the IQ-treated groups.

Table 1. Body and organ weights, and IQ intake

Group	IQ (p.p.m.)	No. rats	Bodyweight (g)	Liver		Average IQ intake	
				Absolute weight (g)	Relative weight (%)	Daily intake (mg/kg b.w.)	Total (mg/kg b.w.)
1	0	240	331 ± 23	9.3 ± 1.7	2.8 ± 0.4	0	
2	0.001	240	332 ± 17	9.1 ± 1.4	2.8 ± 0.4	0.0001	0.008
3	0.01	240	331 ± 19	9.0 ± 1.5	2.8 ± 0.4	0.0007	0.08
4	0.1	240	331 ± 22	8.5 ± 1.2*	2.6 ± 0.3*	0.008	0.9
5	1	240	331 ± 17	8.5 ± 1.2*	2.6 ± 0.3*	0.08	8.7
6	10	240	330 ± 18	9.0 ± 1.3	2.7 ± 0.4	0.76	85.1
7	100	120	319 ± 19*	10.0 ± 1.6*	3.2 ± 0.4*	7.83	877.5

*Significantly different from group 1. IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

The number and size of GST-P-positive foci in rat livers at week 16 is summarized in Table 2. The total numbers of GST-P-positive foci per unit area in the livers in the groups administered 0.001–1 p.p.m. IQ did not differ from the control value (0 p.p.m. group), and no significant increases were observed in any size range of GST-P-positive foci in these groups. Significant increases in the total numbers of GST-P-positive foci per unit area in the liver were observed in the 10 and 100 p.p.m. groups compared with the control. The numbers of GST-P-positive foci composed of 2–4 cells and 5–10 cells in the 10 p.p.m. group and GST-P-positive foci of all sizes in the 100 p.p.m. group were significantly increased.

Formation of IQ-DNA adduct and 8-OHdG in liver DNA. Representative autoradiograms of IQ-DNA adducts in livers are shown in Figure 1. The levels of IQ-DNA adducts in the livers of the 0 and 0.001 p.p.m. IQ-treated groups were under the detectable limit at week 4 (Table 3). IQ-DNA adducts were detectable in the livers of rats administered 0.01 p.p.m. IQ, and adduct formation increased in a dose-dependent manner in groups administered higher doses of IQ. No significant differences in 8-OHdG levels were observed in the liver DNA between any of the groups administered IQ and the control group (Table 3).

Gene expression changes in the liver. Relative mRNA expression of IQ metabolizing genes CYP1A 1and CYP1A2, cell cycle genes PCNA and $p21^{Cip/WAF1}$, p53, and DNA repair genes APE-1 and GADD45 in the livers at week 16 is shown in Figure 2. CYP1A1 was significantly increased in the livers of rats treated with 100 p.p.m. IQ, but not in the lower doses of IQ. CYP1A2, on the other hand, was significantly increased in the 0.01–10 p.p.m. groups, but no significant change was observed in the 100 p.p.m. group. There was no significant difference in the CYP1B1 expression level among groups (data not shown).

A significant increase in PCNA was observed in the 100 p.p.m. group, but not in the groups administered lower doses of IQ, while the negative cell cycle regulator $p21^{Cip/WAF1}$ was significantly induced in the 0.01 p.p.m. group and maximally induced in the 100 p.p.m. group. The expression level of $p21^{Cip/WAF1}$ in the 100 p.p.m. group was significantly higher than in the 10 p.p.m. and lower dose groups. There were no significant changes in p53 expression levels in the IQ-treated groups.

APE-1 was significantly induced in the 10 and 100 p.p.m. groups and GADD45 was significantly induced in the 100 p.p.m. group. IQ had no effect on the expression of Ogg-1, MSH2 or MSH3 (data not shown).

Induction of ACF in the colon. The number and size of ACF in rat colons at week 16 is summarized in Table 4. In the 10 p.p.m. group, the number of ACF composed of one crypt was significantly increased compared with the control. In the 100 p.p.m. group, significant increases were observed in the numbers of all sizes of ACF. In contrast, in the groups administered 0.001–1 p.p.m. IQ, neither the number of any size ACF nor the total number of ACF differed from the control.

Discussion

Dose-response relationships for genotoxic carcinogens have been a topic of intense scientific and public debate. High doses of the genotoxic dietary carcinogen IQ have been demonstrated to induce liver and colon cancers in rats (300 p.p.m. in diet)[8] and liver cancers in nonhuman primates (10 mg/kg b.w./day).[10] However, as the concentrations of IQ in food are generally extremely low,[11] there is uncertainty regarding the carcinogenicity of the doses of IQ to which humans are exposed. The present study shows that IQ at doses of 1 p.p.m. (0.08 mg/kg body weight [b.w.]/day) and lower did not induce either GST-P-positive foci in the liver or ACF in the colon. Only in the groups administered higher doses of IQ, 10 p.p.m. (0.76 mg/kg b.w./day) and 100 p.p.m. (7.83 mg/kg b.w./day), were increases in GST-P-positive foci and ACF observed.

GST-P-positive foci and ACF are well-established preneoplastic lesions of the liver and colon, respectively, in rats. These lesions have been accepted as useful end-point markers in the assessment of carcinogenic effects of environmentally relevant concentrations of carcinogens as they can extend the range of observable effect levels.[24,26] Therefore, the results of the present study suggest the presence of no-effect levels of IQ for both liver and colon carcinogenicity in rats and indicate that the dose-response relationship for carcinogenicity of low dose IQ is nonlinear.

Several threshold mechanisms for genotoxic carcinogens have been suggested, including induction of detoxification processes, cell cycle delay, DNA repair, apoptosis and the suppression of neoplastically transformed cells by the immune system.[12,13,15,33] However, little *in vivo* evidence is available. To explore mechanisms underlying the carcinogenicity of low doses of IQ, we examined the relative mRNA expression of a panel of genes involved in cell proliferation, cell cycle regulation, DNA repair and IQ metabolic activation. We found that the cell proliferation marker PCNA was significantly increased only at a dose of 100 p.p.m., a dose that is carcinogenic. The cell cycle negative regulator $p21^{Cip/WAF1}$, on the other hand, was significantly induced at a dose of 0.01 p.p.m., a dose well below that which induced the formation of preneoplastic lesions. Furthermore, the finding that the levels of $p21^{Cip/WAF1}$ in the groups administered 10 p.p.m. and less were much lower than that of the group administered 100 p.p.m. implies that hepatocytes have adequate capacity to cope with the type of damage that is repaired by the $p21^{Cip/WAF1}$ pathway when exposed to low doses of IQ, but that the repair capacity of these hepatocytes, even in the presence of high $p21^{Cip/WAF1}$ expression, can be overwhelmed when the cell is subjected to very high doses of IQ. It is reasonable to suggest

Table 2. Development of GST-P-positive foci in the livers of rats administered IQ for 16 weeks

Group	IQ (p.p.m.)	No. rats	Size of GST-P positive foci				
			2–4	5–10	11–20	≥21	Total
1	0	240	0.09 ± 0.25	0.03 ± 0.11	0.02 ± 0.11	0.00 ± 0.02	0.15 ± 0.31
2	0.001	240	0.10 ± 0.24	0.04 ± 0.15	0.01 ± 0.07	0	0.16 ± 0.31
3	0.01	240	0.15 ± 0.47	0.07 ± 0.41	0.02 ± 0.22	0.02 ± 0.03	0.26 ± 1.30
4	0.1	240	0.10 ± 0.28	0.04 ± 0.15	0.01 ± 0.07	0.01 ± 0.08	0.15 ± 0.35
5	1	240	0.10 ± 0.25	0.04 ± 0.16	0.01 ± 0.06	0	0.14 ± 0.33
6	10	240	0.51 ± 0.65	0.19 ± 0.36*	0.02 ± 0.10	0.01 ± 0.11	0.74 ± 0.88*
7	100	120	26.23 ± 18.24*	23.81 ± 16.23*	19.25 ± 11.70*	18.74 ± 11.81*	88.03 ± 50.41*

*Significantly different from group 1. GST-P, glutathione *S*-transferase placental form positive foci; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

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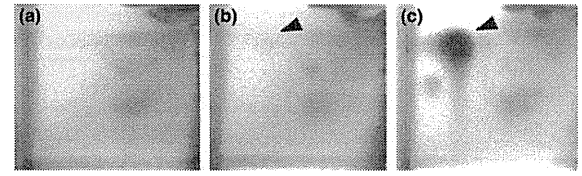

Fig. 1. Autoradiograms of 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ)-DNA adducts the livers of 0 (a), 0.001 (b) and 100 (c) p.p.m. IQ-treated groups at week 4. Arrowheads indicate IQ-DNA adduct. The imaging plates were exposed for 3 h (a) and 24 h (b and c).

Table 3. IQ-DNA adduct and 8-OHdG formation in liver DNA

Group	IQ (p.p.m.)	No. rats	Adduct level ($\times10^{-7}$ ntd)	8-OHdG ($\times10^{-5}$ dG)
1	0	5	UDL	0.23 ± 0.07
2	0.001	5	UDL	0.25 ± 0.05
3	0.01	5	0.045 ± 0.02	0.24 ± 0.07
4	0.1	5	0.1 ± 0.004	0.32 ± 0.10
5	1	5	1.7 ± 0.07	0.24 ± 0.08
6	10	5	12.7 ± 0.07	0.22 ± 0.07
7	100	5	107.0 ± 0.07	0.23 ± 0.08

IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; ntd, nucleotide; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; UDL, under the detectable limit.

that suppression of cell cycle progression by $p21^{Cip/WAF1}$ followed by DNA repair is at least one of the mechanisms responsible for the observed no-effect of low doses of IQ in rats in the present model.

It is known that the vast majority of DNA damage is repaired by base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR).[34] APE-1 plays an essential role in the BER repair process by cleaving the phosphodiester backbone.[35] The activities of two different heterodimeric complexes, MSH2-MSH3 and MSH2-MSH6, belonging to the MMR system are mainly responsible for the post-replicative repair of mismatches.[36] We found that IQ significantly increased the expression levels of APE-1 but not MSH2 and MSH3 at doses of 10 and 100 p.p.m. in the liver. It has also been reported that IQ has no effect on expression of ERCC1, which is a key molecule in the NER process.[37] These findings suggest that BER rather than MMR or NER responds to IQ-induced DNA damage.

GADD45 is involved in a variety of growth regulatory mechanisms, including DNA repair, growth arrest and apoptosis.[38] It is induced by genotoxic and certain other cell stresses by p53-dependent and independent pathways.[39,40] GADD45 expression was significantly induced in the 100 p.p.m. group. The fact that significant induction of APE-1 and GADD45 was observed only at the highest doses of 10 and/or 100 p.p.m. indicate the IQ-induced DNA damage response is dose-dependent. Moreover, the fact that in the groups with low doses expression of APE-1 and GADD45 were not affected and that there was a significant but moderate induction of $p21^{Cip/WAF1}$ imply that normal physiological levels of these genes are sufficient to repair the DNA damage caused by low doses of IQ. However, the expression levels of these genes are all increased by higher carcinogenic doses of IQ. A reasonable explanation of the no-effect of low doses of IQ and the carcinogenicity of high doses of IQ is that carcinogenicity is the consequence of a disruption in the balance between DNA damage and repair and between abnormal cell proliferation and apoptosis or cell cycle regulation.

Our results show that p53 gene expression is not induced by administration of IQ. Furthermore, p53-deficient mice do not show higher susceptibility to IQ-induced liver carcinogenesis than wild type mice.[41] These results suggest that p53 does not have a significant impact on the carcinogenicity of IQ.

DNA adduct formation by metabolic activation of IQ is believed to play an important role in the carcinogenicity of IQ.[42] Formation of IQ-DNA adducts in the liver showed a linear dose-dependency and proved to be one of the most sensitive end-points for the detection of exposure to IQ. Adduct formation was detectable in groups administered far lower doses of IQ compared with detection of other end-points such as cell proliferation and preneoplastic lesion induction. That IQ-DNA adduct formation was not detected in the 0.001 p.p.m. group was most likely due to the detection limit of the assay. It should be noted that DNA adduct is a premutagenic lesion and not necessarily correlated to the frequencies of mutation and cancer induced by genotoxic compounds. For example, it is known that IQ forms DNA adducts in the kidneys and stomach of both rats and monkeys, but does not induce tumors in these organs.[43,44] Our present findings of a linear dose-response of IQ-DNA adduct formation and a nonlinear carcinogenic dose-response to IQ administration support the idea that IQ-DNA adducts do not necessarily lead to mutation and formation of cancerous lesions. Our results are also in line with previous results on HCA including MeIQx[1,18,45] and PhIP.[24] These results can be explained, at least in part, by the actions of gene products such as $p21^{Cip/WAF1}$, GADD45 and APE-1 and the other repair genes for DNA damage. Moreover, in the case of MeIQx, it has been suggested that formation of DNA adducts alone might not be sufficient to produce cancers and that the MeIQx-induced genetic alterations in the liver are enhanced by liver regeneration induced by high doses of MeIQx itself.[1] Therefore, while IQ-DNA adduct formation is important in IQ carcinogenicity, high levels of adduct formation are likely required and other factors such as cell proliferation can affect the balance between DNA damage and repair and lead to fixation of DNA mutations into the cell's genome.

It has been demonstrated *in vitro* that IQ is more efficiently metabolized and activated by CYP1A2 than by CYP1A1 or CYP1B1.[46] However, limited *in vivo* data are available. In a study by McPherson *et al.*[47], no significant induction in mRNA expression level or activity of either CYP1A1 or CYP1A2 were reported in the livers of rats receiving 300 p.p.m. IQ in the diet for 52 weeks, but these enzymes were significantly increased after daily administration of 20 mg/kg b.w. IQ by oral gavage for 3 days; in the average adult rat, a dose of 300 p.p.m. IQ in the diet is approximately equivalent to administration of 20 mg/kg b.w. IQ by oral gavage. The results of the present study revealed that IQ significantly induced CYP1A2 expression at doses from 0.01 to 10 p.p.m., but CYP1A2 was not induced in the 100 p.p.m. group. The lack of effect of 100 p.p.m. IQ on CYP1A2 expression is consistent with the results in rats receiving 300 p.p.m. IQ in the diet for 52 weeks.[47] Significant increases in CYP1A1 expression in the 100 p.p.m. group provide an alternative mechanism that can compensate for decreased CYP1A2 activity. However, as noted above, in apparent contrast to our results, in the study by McPherson *et al.*,[47] administration of 300 p.p.m. IQ over the course of 52 weeks did

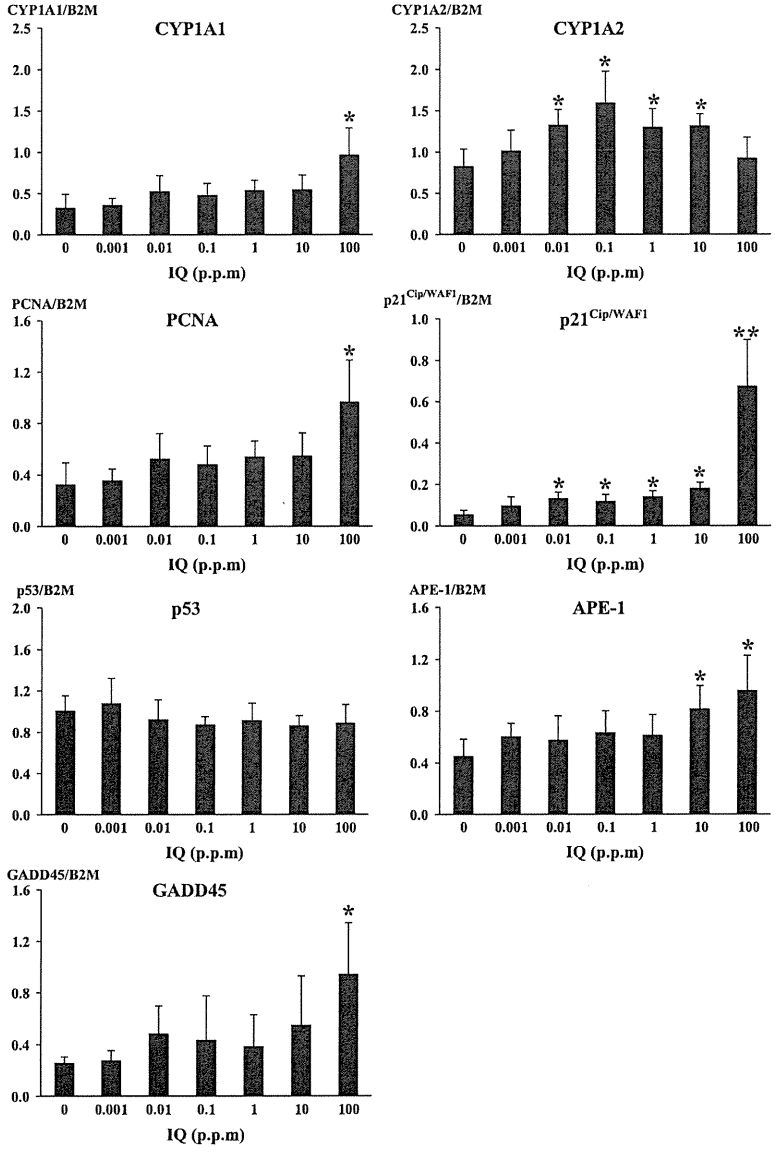

Fig. 2. Relative mRNA expression in the livers of rats at week 16. *Significantly different from 0 p.p.m. **Significantly different from all other groups. APE-1, AP endonuclease-1; B2M, beta-2-microglobulin; GADD45, growth arrest and DNA damage-inducible protein 45; PCNA, proliferating cell nuclear antigen.

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Table 4. Development of ACF in the colons of rats administered IQ for 16 weeks

Group	IQ (p.p.m.)	No. rats	Size of ACF				
			1	2	3	≥4	Total
1	0	240	0.08 ± 0.28	0.12 ± 0.32	0.06 ± 0.25	0.08 ± 0.29	0.33 ± 0.64
2	0.001	240	0.12 ± 0.36	0.08 ± 0.29	0.10 ± 0.32	0.09 ± 0.30	0.39 ± 0.69
3	0.01	240	0.15 ± 0.41	0.15 ± 0.42	0.06 ± 0.24	0.06 ± 0.24	0.43 ± 0.77
4	0.1	240	0.11 ± 0.33	0.11 ± 0.35	0.06 ± 0.25	0.08 ± 0.27	0.36 ± 0.63
5	1	240	0.15 ± 0.45	0.10 ± 0.30	0.10 ± 0.33	0.05 ± 0.23	0.41 ± 0.80
6	10	240	0.19 ± 0.48*	0.16 ± 0.41	0.07 ± 0.25	0.09 ± 0.40	0.50 ± 0.86
7	100	120	1.48 ± 1.46*	1.29 ± 1.51*	0.70 ± 0.93*	0.72 ± 1.01*	4.19 ± 3.34*

*Significantly different from group 1. ACF, aberrant crypt foci; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

not induce CYP1A1. Therefore, it is reasonable to postulate that the dose-relationship between IQ and induction of CYP1A1 is not a simple dose-response. CYP1B1 does not appear to be involved in the metabolism of IQ at doses up to 100 p.p.m. in rats. The findings described above demonstrate the importance of taking into account dosage, duration and route of exposure in interpretation of the data on metabolic activation of IQ. Further studies on the dose-response relationships between chronic IQ exposure and the protein expression levels and activities of detoxifying enzymes, especially at doses relevant to human exposure, would provide further insight into the role of metabolic activation in IQ carcinogenicity.

Oxidative DNA damage does not appear to play a role in IQ-induced carcinogenesis. In the present study, no significant changes in 8-OHdG levels or Ogg1 expression levels in the livers of IQ-treated rats were observed. Our results are consistent with the recent findings in IQ-treated Big Blue rats that oxidative stress was not responsible for the initiation of IQ-induced carcinogenesis in the liver and colon.[37] In this respect, IQ is different from MeIQx, in which oxidative DNA damage plays an important role in liver carcinogenesis.[48]

In summary, the present study provides the first experimental data on the carcinogenicity of low doses of IQ in both the liver and colon of the test animal and compares the effect of IQ at the cellular level with its carcinogenic effect. Our findings support the idea that there is a practical threshold that should be considered when evaluating the risk of genotoxic carcinogens. To this end, further accumulation of data, especially mechanistic data, should be promoted to facilitate not only an understanding of the carcinogenic effects of low doses of genotoxic carcinogens but also to establish an accurate means of quantitative risk assessment.

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Disclosure Statement

The authors have no conflict of interest.

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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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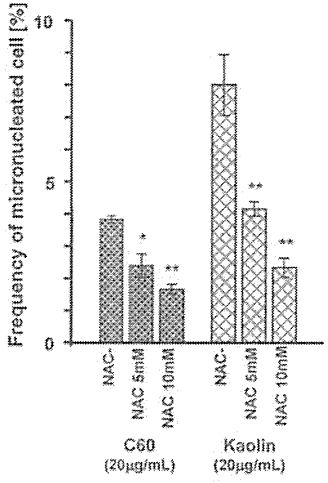

Fig. 1. Effects of anti-oxidative agents on the micronucleus inducing activity of nanoparticles. Values represent the means of three experiments ± SD. Asterisks (*, ** for $p<0.05$ and $p<0.01$, respectively) indicate significant differences from cells without NAC in the Student's t-test. Concentrations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

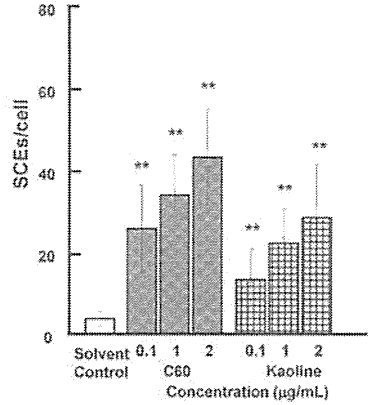

Fig. 2. Sister chromatid exchange (SCE) in CHO AA8 cells following treatment with C60 or kaolin for 1 h. The values represent the means of three experiments ± SD. Asterisks (**) indicate a significant difference ($p<0.01$) from control (treatment with 0.005% (v/v) Tween-80) cells in the Student's t-test.

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

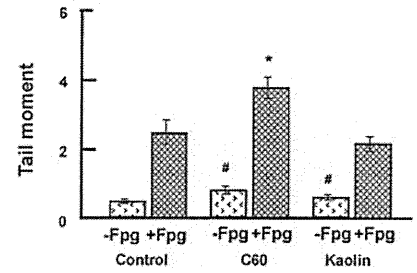

Fig. 3 DNA damage measured by comet assay in lungs of C57BL/6J mice intratracheally instilled with particles, with or without FPG treatment. Male mice were treated at a dose of 0.2 mg of particles per animal, and sacrificed 3 h after particle administration. The values represent the means of data for 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-