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 m^2) and all-cause mortality is well known (1,2). The World Health Organization defines overweight as a BMI of 25.0–29.9 kg/m^2 and obesity as a BMI of ≥30 kg/m^2. 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

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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.

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,

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.

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 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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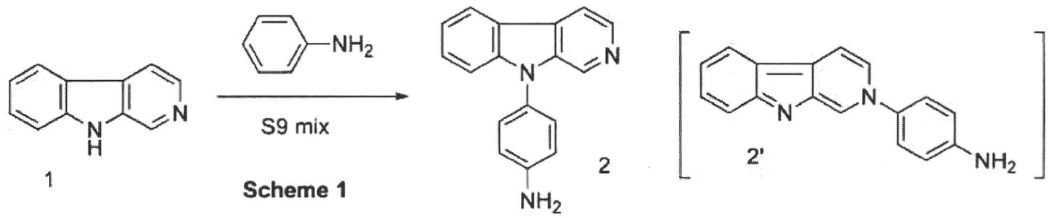
Abstract – 9-(4'-Aminophenyl)-9*H*-pyrido[3,4-*b*]indole **2** is a mutagenic compound produced by non-mutagenic norharman **1** and aniline in the presence of S9 mix. 9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole **4**, the relay compound for synthesis of **2**, was synthesized starting from ethyl indole-2-aldehyde **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**.

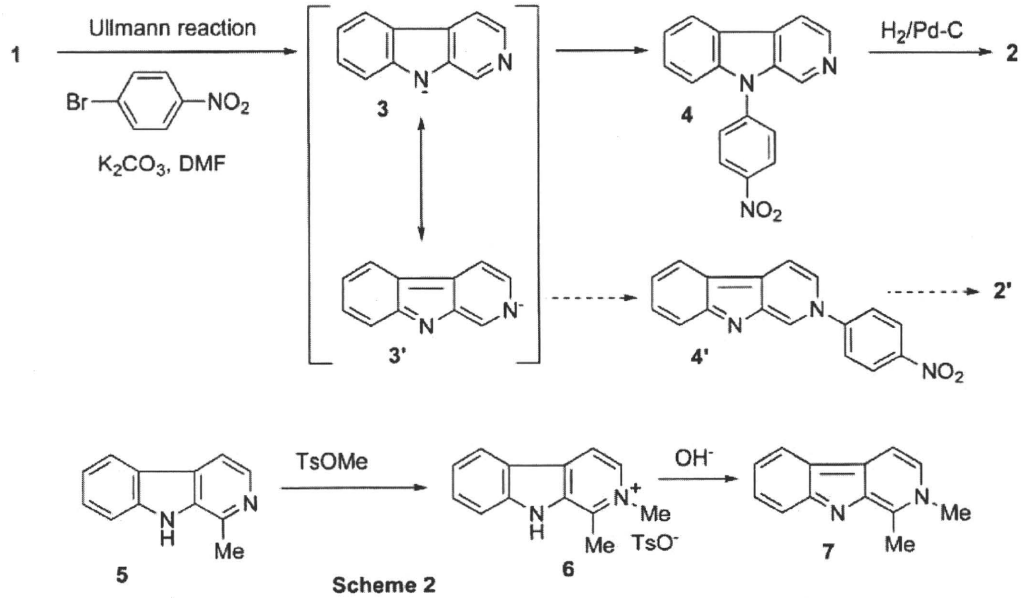
INTRODUCTION

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**.

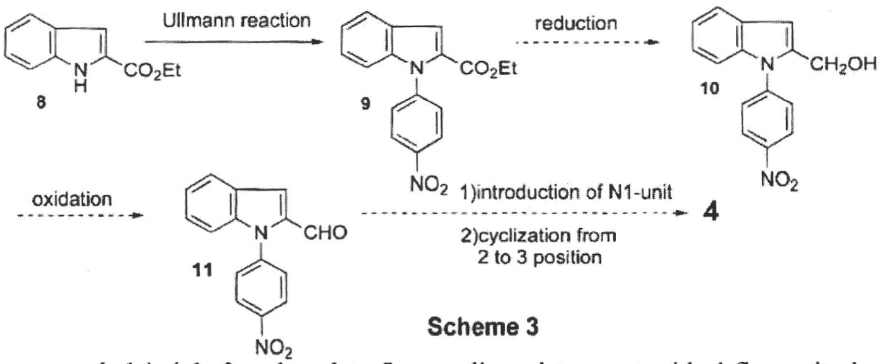


Scheme 2

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.

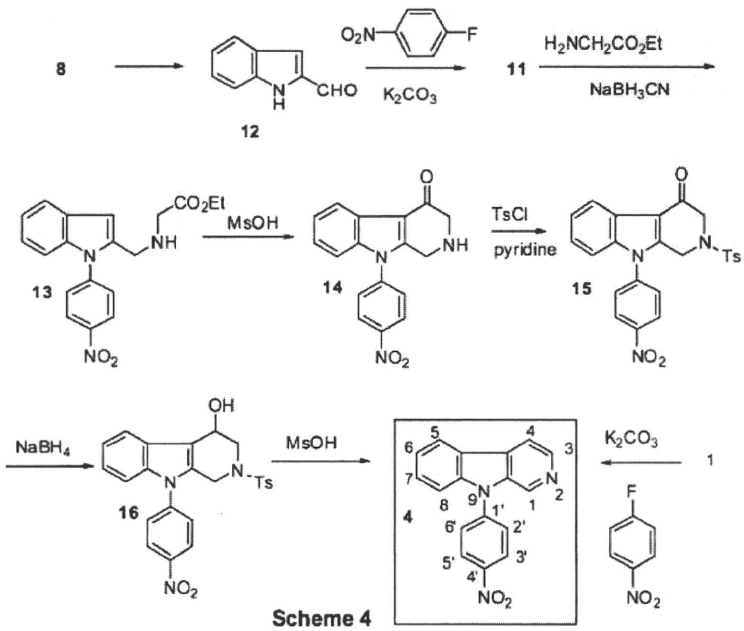


Scheme 3

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.

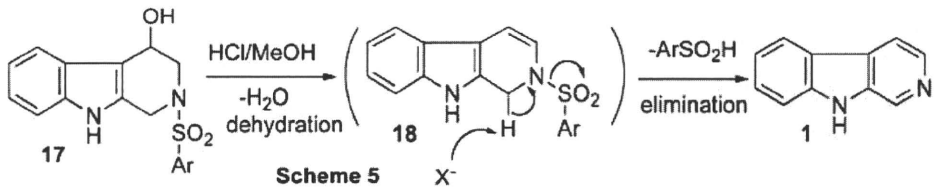
The first *N*-(4-nitro)phenylation of indole-2-carboxaldehyde[5] **12** prepared from **8**, which we feared to proceed with difficulty due to the sensitive reactivity of aldehyde functionality, went much better than expected (51% yield). The *N*-(4'-nitrophenyl)indole-2-carboxaldehyde **11** thus obtained 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, -$CH_2C\underline{H}_3$), 3.41 (2H, s, -CH_2NH-), 3.94 (2H, s, -NCH_2CO-), 4.12 (2H, *J*=8.0 Hz, -$OC\underline{H}_2CH_3$), 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 $C_{24}H_{21}N_3O_5S$: 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, -$CH_2C\underline{H}_3$), 4.25 (2H, t, *J*=8.0 Hz, -$OC\underline{H}_2CH_3$), 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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