TABLE 3
HRs and 95% CIs of gastric cancer according to quartile of energy-adjusted intake of isoflavone (genistein), miso soup, and soy food among women[1]

			All gastric cancer			Upper third, including cardia		Distal	
Quartile	Median	Person-years	No. of cases	HR1 (95% CI)[2]	HR2 (95% CI)[3]	No. of cases	HR2 (95% CI)[3]	No. of cases	HR2 (95% CI)[3]
Isoflavone (genistein) (mg/d)									
First	9.4	106,951	74	1.00 (reference)	1.00 (reference)	7	1.00 (reference)	46	1.00 (reference)
Second	17.3	109,818	83	1.03 (0.75, 1.41)	1.08 (0.78, 1.49)	6	0.72 (0.24, 2.20)	58	1.14 (0.77, 1.70)
Third	26.0	110,797	102	1.16 (0.85, 1.58)	1.23 (0.90, 1.70)	7	0.78 (0.26, 2.35)	75	1.33 (0.90, 1.97)
Fourth	41.8	110,399	91	0.99 (0.71, 1.37)	1.07 (0.77, 1.50)	13	1.43 (0.52, 3.95)	58	1.00 (0.66, 1.53)
P-trend				0.9	0.6		0.4		0.9
Isoflavone (genistein) from fermented soy food (g/d)[4]									
First	3.0	105,253	77	1.00 (reference)	1.00 (reference)	6	1.00 (reference)	48	1.00 (reference)
Second	8.0	110,124	80	0.86 (0.62, 1.19)	0.90 (0.65, 1.25)	7	0.76 (0.24, 2.37)	56	0.93 (0.62, 1.39)
Third	14.1	112,341	86	0.81 (0.57, 1.13)	0.87 (0.61, 1.23)	9	0.83 (0.26, 2.59)	63	0.90 (0.59, 1.37)
Fourth	25.6	110,247	107	0.91 (0.65, 1.28)	1.00 (0.71, 1.42)	11	0.89 (0.28, 2.80)	70	0.93 (0.61, 1.43)
P-trend				0.7	0.9		0.9		0.8
Isoflavone (genistein) from nonfermented soy food (g/d)[5]									
First	3.2	107,879	85	1.00 (reference)	1.00 (reference)	10	1.00 (reference)	53	1.00 (reference)
Second	6.5	109,703	87	1.02 (0.76, 1.38)	1.07 (0.79, 1.45)	7	0.71 (0.27, 1.91)	60	1.14 (0.79, 1.66)
Third	10.7	110,224	97	1.14 (0.85, 1.53)	1.20 (0.89, 1.61)	7	0.77 (0.28, 2.08)	69	1.29 (0.89, 1.86)
Fourth	20.6	110,159	81	0.99 (0.73, 1.35)	1.03 (0.75, 1.42)	9	1.06 (0.41, 2.70)	55	1.07 (0.72, 1.58)
P-trend				0.9	0.7		0.9		0.6
Miso soup (mL/d)									
First	47	104,994	92	1.00 (reference)	1.00 (reference)	6	1.00 (reference)	62	1.00 (reference)
Second	140	106,895	84	0.80 (0.59, 1.08)	0.85 (0.63, 1.14)	10	1.59 (0.57, 4.46)	49	0.70 (0.48, 1.02)
Third	244	111,927	92	0.79 (0.59, 1.07)	0.81 (0.59, 1.11)	9	1.04 (0.35, 3.15)	69	0.84 (0.58, 1.22)
Fourth	384	114,148	82	0.67 (0.49, 0.92)	0.71 (0.50, 1.01)	8	0.83 (0.25, 2.76)	57	0.69 (0.45, 1.05)
P-trend				0.02	0.06		0.6		0.2
Soy food (g/d)[6]									
First	33.6	106,148	84	1.00 (reference)	1.00 (reference)	8	1.00 (reference)	52	1.00 (reference)
Second	58.7	109,310	86	0.94 (0.69, 1.27)	0.99 (0.73, 1.35)	6	0.65 (0.22, 1.91)	59	1.04 (0.71, 1.52)
Third	85.2	111,361	99	1.05 (0.78, 1.41)	1.12 (0.83, 1.53)	10	1.09 (0.41, 2.90)	71	1.21 (0.83, 1.76)
Fourth	141.0	111,146	81	0.92 (0.67, 1.27)	0.99 (0.71, 1.38)	9	1.10 (0.39, 3.08)	55	1.02 (0.68, 1.53)
P-trend				0.8	0.8		0.6		0.8

[1] Cox proportional hazards models were used.
[2] HR adjusted for age and public center area.
[3] HR further adjusted for BMI, smoking status, ethanol intake, family history of gastric cancer, vegetable intake, fruit intake, fish intake, salt intake, and total energy intake.
[4] The consumption of miso (for miso soup) and *natto*.
[5] The consumption of soymilk, tofu for miso soup, tofu for other dishes, *yushidofu*, *koyadofu*, and *aburaage*.
[6] Total of fermented and nonfermented soy food.

Several limitations of the study warrant mention. First, because we assessed isoflavone intake by using an FFQ, some misclassification of isoflavone intake may have arisen when the effect on GC risk was estimated. Such misclassification was likely nondifferential and would tend to result in an underestimation of the effect of isoflavone intake. Second, we did not collect information on isoflavone supplement use. However, a relatively recent 2006 survey on supplement use in Japan showed a low prevalence of isoflavone supplementation (<1.6%) (42); thus, intake from supplements is considered to be negligible. Third, it was not possible to distinguish hormone replacement therapy from oral contraceptives. This may have confounded any possible effect, particularly among those participants in menopause. Finally, we were unable to adjust for *H. pylori* infection. However, because we showed a high infection rate based on CagA and IgG positivity in an earlier published subset of the JPHC study participants, 99% among GC case and 90% among control (43), most participants could be regarded as being infected, and the difference of infection likely did not affect the results.

In conclusion, the current study found no evidence to support the hypothesis that higher intakes of isoflavone prevent GC in either men or all women. However, we did observe associations suggestive of a higher risk with isoflavone intake in women with EFH use. Our findings warrant further investigation.

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TABLE 4
HRs and 95% CIs of gastric cancer according to quartile of energy-adjusted intake of isoflavone (genistein), miso soup, and soy food by exogenous female hormones[1]

	EFH never user (n = 36,930)			EFH ever user (n = 5853)			
Quartile	Person-years	No. of cases	HR (95% CI)[2]	Person-years	No. of cases	HR (95% CI)[2]	P-interaction
Isoflavone (genistein)							
First	86,437	65	1.00 (reference)	13,906	5	1.00 (reference)	
Second	89,308	67	0.96 (0.68, 1.37)	14,593	7	1.25 (0.38, 4.06)	
Third	89,947	86	1.13 (0.80, 1.59)	15,823	11	1.78 (0.58, 5.47)	
Fourth	88,627	69	0.89 (0.61, 1.29)	16,203	17	2.80 (0.93, 8.39)	
P-trend			0.7			0.03	0.04
Isoflavone (genistein) from fermented soy food (g/d)[3]							
First	85,111	63	1.00 (reference)	13,267	6	1.00 (reference)	
Second	90,196	66	0.87 (0.60, 1.25)	14,354	9	1.22 (0.41, 3.66)	
Third	89,954	74	0.87 (0.59, 1.27)	16,833	7	0.78 (0.23, 2.60)	
Fourth	89,058	84	0.91 (0.62, 1.34)	16,071	18	2.02 (0.69, 5.97)	
P-trend			0.7			0.2	0.2
Isoflavone (genistein) from nonfermented soy food (g/d)[4]							
First	86,891	75	1.00 (reference)	14,037	5	1.00 (reference)	
Second	89,328	75	1.04 (0.75, 1.43)	15,254	6	1.17 (0.35, 3.91)	
Third	89,437	72	0.99 (0.41, 1.37)	15,712	18	3.27 (1.18, 9.12)	
Fourth	88,662	65	0.94 (0.67, 1.33)	15,522	11	2.05 (0.68, 6.18)	
P-trend			0.7			0.07	0.051
Miso soup							
First	85,458	79	1.00 (reference)	13,880	8	1.00 (reference)	
Second	87,746	65	0.74 (0.53, 1.04)	14,031	9	1.01 (0.38, 2.69)	
Third	90,907	76	0.75 (0.53, 1.05)	15,616	13	1.44 (0.54, 3.86)	
Fourth	90,207	67	0.65 (0.45, 0.96)	16,998	10	1.01 (0.33, 3.05)	
P-trend			0.04			0.8	0.62
Soy food[5]							
First	86,192	75	1.00 (reference)	13,577	4	1.00 (reference)	
Second	89,507	70	0.87 (0.62, 1.22)	14,622	7	1.69 (0.48, 5.94)	
Third	89,735	80	0.98 (0.70, 1.37)	16,006	14	3.20 (0.99, 10.3)	
Fourth	88,885	62	0.83 (0.58, 1.19)	16,319	15	3.76 (1.14, 12.4)	
P-trend			0.5			0.01	0.02

[1] Cox proportional hazards models were used. EFH, exogenous female hormones.
[2] Adjusted for age, public center area, BMI, smoking status, ethanol intake, family history of gastric cancer, vegetable intake, fruit intake, fish intake, salt intake, total energy intake, and menopausal status.
[3] The consumption of miso (for miso soup) and *natto*.
[4] The consumption of soymilk, tofu for miso soup, tofu for other dishes, *yushidofu*, *koyadofu*, and *aburaage*.
[5] Total of fermented and nonfermented soy food.

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Combined impact of five lifestyle factors and subsequent risk of cancer: The Japan Public Health Center Study

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ABSTRACT

Objective. To evaluate whether 5 combined healthy lifestyle factors (not smoking, moderate drinking, eating minimum salt-preserved foods, being physically active, and having appropriate body mass index) are associated with reduced risk of cancer.

Methods. Participants were enrolled in the Japan Public Health Center Study and responded to the 5-year follow-up questionnaire covering lifestyle factors in 1995–1999 at ages 45–74 years. During follow up through December 31, 2006, 3451 and 2125 cases of cancer were newly identified in men and women, respectively. For men and women, a factor-dependent risk reduction was observed for healthy lifestyles and cancer development. Compared to 0–1 healthy lifestyle factors, the adjusted RRs and 95% CIs for adherence to 2, 3, 4, and 5 healthy factors were 0.86 (0.78–0.95), 0.72 (0.65–0.80), 0.61 (0.54–0.69), and 0.57 (0.45–0.72), respectively, for men (P for trend<0.0001) and 0.86 (0.53–1.40), 0.73 (0.46–1.16), 0.68 (0.42–1.08), and 0.63 (0.39–1.01), respectively, for women (P for trend = 0.0003). Risk was reduced 14% and 9% by each one healthy lifestyle for men and women, respectively. Risk reduction was more pronounced among elderly women.

Conclusion. These combined lifestyle factors have a considerable impact on preventing cancer.

Introduction

Lifestyle factors such as cigarette smoking, alcohol consumption, diet, physical activity, and relative weight (body mass index [BMI]) are considered important core factors for preventing cancer (Secretan et al., 2009; World Cancer Research Fund/American Institute for Cancer Research, 2007). In fact, "Cancer Prevention for Japanese," one of the cancer prevention guidelines existing in Japan, which was proposed based on systematic review of epidemiologic studies by the Research Group for the Development and Evaluation of Cancer Prevention strategies in Japan (the 3rd term of 10-year comprehensive strategy for cancer control from the Ministry of Health, Labor and Welfare (MHLW)) (National Cancer Center [NCC web site], 2003: http://epi.ncc.go.jp/can_prev/93/180.html), presents these factors as recommended key aspects for cancer prevention, in addition to checking the status of infection. Although the individual effect of these factors on cancer has been well documented, their combined effect is not well known.

Based on projection models, we have shown that lifestyle factors of current smoking, excessive alcohol drinking, and obesity are suggested as common risk factors for cancer and cardiovascular disease (CVD), particularly in men (Tanaka and for the JPHC Study Group, 2009).

In this study that considered more factors, we evaluated the extent to which the combined 5 lifestyle factors are associated with reduced risk of cancer in a Japanese population.

Methods

Study population

Details of the study design have been described previously (Tsugane and Sobue, 2001). The participants in the present study were Japanese residents included in the Japan Public Health Center (JPHC)-Based Prospective Study who responded to the 5-year follow-up questionnaire covering lifestyle factors during 1995–1999 at ages 45–74 years. Subjects were followed from the starting point for the present analysis (1995–1999) until December 31, 2006. The institutional review board of the National Cancer Center, Tokyo, Japan, approved the study. We identified 133,323 subjects as the study population.

Follow-up and identification of cancer cases

Residency registration and death registration are required by the Basic Residential Register Law and Family Registry Law, respectively, and the registries are thought to be complete. During the follow-up period in the present study, 11,073 subjects died, and 4904 moved away from the study area, and were lost to follow-up.

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We identified incident data for cancer by active patient notification from major local hospitals in the study area and from data linkage with population-based cancer registries. We coded cancer cases according to the *International Classification of Diseases for Oncology*, 3rd edition (World Health Organization, 2000). In our cancer registry system, the proportion of cases for which information was available from death certificates only was 6.1%.

Questionnaire

The questionnaire covered sociodemographic characteristics, medical history, smoking and drinking habits, diet, physical activity, and so on. The validated FFQ asked subjects about their usual intake of food during the previous year in standard portions/units and 9 frequency categories. (Tsubono et al., 1996). Salt-preserved foods and their salt content included in the questionnaire were: pickled vegetables (1.5–7.6%), dried and salted fish (1.7–4.1%), salted fish roe *tarako* (salted Alaska pollack roe) or suziko (salted salmon roe) (4.6–4.8%), and miso soup (1%) (Resource Council, 2002).

Subjects were asked about the average amount of time spent per day in three types of physical activity: heavy physical work or strenuous exercise, sitting, and standing or walking. MET-h/d were estimated by multiplying the daily time score for each activity by the MET intensity of that activity (Ainsworth et al., 2000; Inoue et al., 2008).

After excluding subjects with non-Japanese nationality (51), emigration occurring before the starting point (183), ineligibility due to incorrect birth date (7), or duplicate enrollment (4), we established a population-based cohort of 133,076 subjects. After exclusion of 12,067 subjects who had died (3432), moved away from the study area (8231), or been lost to follow-up before the starting point (304), and 121,009 eligible subjects remained. Among them, 98,456 responded to the questionnaire, yielding a response rate of 81.4%. We excluded subjects who had been diagnosed or reported as having cancer (2156), CVD (1188), or ischemic heart disease (1471) before the starting point, who had missing data regarding smoking (4702), alcohol (2439), physical activity (4490), missing or inadequate data for BMI (2822), or who reported extreme total energy intake (upper 2.5% or lower 2.5% for each sex). The final analysis included 78,548 subjects (36,964 men and 41,584 women).

Scores for lifestyle factors

The participants were regarded as adhering to the healthy lifestyle if they were nonsmokers (never smoked), drinking in moderation (drinking with <150 g/week), eating minimum salt-preserved foods (eating fish roe <0.67 g/week), being physically active (≥37.5 metabolic equivalents [MET]-h/d for men and ≥31.9 MET-h/d for women), having an adequate BMI (within 21–27 for men and 19–25 for women). The threshold was identified for each factor by means stated in the guideline or by the means used in our previous observation (Inoue et al., 2008; Takachi et al., 2010). Cut-off points for BMI were derived from the guideline, based on Japanese data representative at the time the guideline was reported (Inoue et al., 2004a). Based on our previous report, salted fish roe, but neither other salt-preserved foods nor total sodium intake, was associated with total cancer (Takachi et al., 2010). Pickled vegetables, dried and salted fish, and miso soup also include vitamins, n-3 fatty acids, and isoflavone, respectively, which may have beneficial effects on cancer. However, the elevated risk of total cancer by salted fish roe might be explained by destruction of the mucosal barrier by a high salt concentration, which leads to inflammation, diffuse erosion, and degeneration (Takachi et al., 2010). In the report, elevated risk was observed above the 4th quintile group, which corresponds to >0.67 g/d in the present data set. This corresponds to eating salted fish roe at a portion size once a month. Similarly, we have reported reduced risk of total cancer among the highest quartile group of total MET-h/d (Inoue et al., 2008). This corresponds to ≥37.5 MET-h/d for men and ≥31.9 MET-h/d for women in the present data set. For example, heavy physical work or strenuous exercise ≥1 h, sitting ≥8 h, and standing or walking ≤1 h can be calculated as 37.5 MET-h/d. This is typical for office workers with some active daily activity added. Similarly, no heavy physical work or strenuous exercise, sitting ≤3 h, and standing or walking ≤3 h is calculated to 31.4 MET-h/d, which is typical of the daily activity of housekeepers. We combined past smokers with current smokers because their risk of developing cancer was similar to current smokers, especially in women, based on our previous report (Inoue et al., 2004b). The general approach consisted of attributing a score of 1 to subjects who met the criteria for healthy lifestyle and 0 for not. Table 1 summarizes the definition of adherence to each lifestyle factor and the distribution by gender. More than 90% of women adhered to not smoking and moderate drinking, whereas fewer than 50% of the men did so. Due to the big difference in distribution of adherence to healthy lifestyle factors by gender, all the analyses were conducted separately for men and women.

We also conducted a weighted score approach based on factor response observed in our cohort previously. Coefficients of relative risks (RRs) derived from our previous publication regarding each lifestyle factor (Inoue et al., 2004a,b, 2005, 2008; Takachi et al., 2010) were multiplied by 10 and summed to present a risk score (Steyerberg, 2009).

Statistical analysis

We calculated person-years of follow-up for each subject from the starting point to the date of cancer diagnosis, date of emigration from the study area, date of death, or end of the follow-up (December 31, 2006), whichever came first. RRs and 95% confidence intervals (CIs) of developing cancer were estimated by 5 lifestyle factors individually and in combination. We used Cox proportional hazards models with adjustment for potential confounding variables such as age in years (<49, 50–54, 55–59, 60–64, 65–69, and >70), public health center area, and past history of diabetes mellitus (yes/no) by the SAS PHREG procedure (SAS Institute, Cary, NC). In the report on salt or salt-preserved foods from this cohort, the public health center area was not adjusted because consumption of these foods was defined by area, and adjustment masked their true influence on cancer (Takachi et al., 2010). Therefore, the public health center area was not adjusted in the individual analysis for salt-preserved food (Table 2).

We calculated population attributable fraction (PAF) (Miettinen, 1974) that might be associated with lack of adherence to all the recommendations, that is, the preventable fraction when all subjects would have adhered to all 5 healthy lifestyle factors as:

$$PAF_{ALL} = \sum P_i[HR_i - HR_5]\}/(1 \times HR_5 + \sum P_i[HR_i - HR_5]) \times 100,$$

and furthermore, the preventable fraction when subjects would have adhered to one additional healthy lifestyle for the entire cohort except the healthiest group was calculated as

$$PAF_{+1} = \sum P_i[HR_i - HR_{i+1}]\}/(1 \times HR_5 + \sum P_i[HR_i - HR_{i+1}]) \times 100,$$

Table 1
Adherence to 5 lifestyle factors among 78,548 Japanese subjects (1995).

Healthy lifestyle factors	Men (n=36,964)	Women (n=41,584)
Nonsmoking		
0 (current smoking or past smoking)	24,205 (65.5%)	2821 (6.8%)
1 (never smoking)	12,759 (34.5%)	38,763 (93.2%)
Moderate drinking		
0 (drinking alcohol ≥150 g/week)	18,658 (50.5%)	1252 (3.0%)
1 (drinking alcohol <150 g/week)	18,306 (49.5%)	40,332 (97.0%)
Eat minimum salt-preserved foods		
0 (eating fish roe at ≥0.67 g/d)[a]	7834 (21.2%)	8997 (21.6%)
1 (eating fish roe at 0–0.67 g/d)[a]	29,130 (78.8%)	32,587 (78.4%)
Being physically active		
0 (men, <37.5 MET-h/d[b]; women, <31.9 MET-h/d[c])	27,752 (75.1%)	23,981 (57.7%)
1 (men, ≥37.5 MET-h/d[b]; women, ≥31.9 MET-h/d[c])	9212 (24.9%)	17,603 (42.3%)
Maintaining adequate/appropriate BMI		
0 (men, <21, ≥27; women, <19, ≥25)	10,929 (29.6%)	14,104 (33.9%)
1 (men, 21–27; women, 19–25)	26,035 (70.4%)	27,480 (66.1%)

[a] The amount 0.67 g/d corresponds to eating salted fish roe at a portion size (quarter of *tarako* (Alaska pollack roe; salt content 4.6–4.8%) which is about 20 g) once a month.
[b] Physical activity level of 37.5 MET-h/d corresponds to for example, typical type of office worker with some active daily activity added. (Heavy physical work or strenuous exercise>=1 h, sitting>=8 h, and standing or walking <1 h).
[c] Physical activity level of 31.9 MET-h/d corresponds to for example, typical type of daily activity of house keeper. (Heavy physical work or strenuous exercise none, sitting <3 h, and standing or walking 3<=h can be calculated to be 31.4 MET-h/d).

Table 2
Independent relative risk (RR) of cancer incidence for individual healthy lifestyle factors among Japanese subjects (1995–2006).

Lifestyle factor	Men		Women	
	RR (95% CI)[a]	P	RR (95% CI)	P
Nonsmoking versus current smoking (including past smoking)	0.67 (0.62–0.72)	<0.0001	0.76 (0.64–0.90)	0.002
Moderate drinking versus not moderate (≥150 g/week) drinking	0.89 (0.83–0.96)	0.001	0.92 (0.70–1.21)	0.56
Eating minimum salt-preserved foods versus not	0.91 (0.84–0.96)	0.02	0.94 (0.85–1.04)	0.20
Physically active versus inactive	0.88 (0.81–0.95)	0.002	0.87 (0.80–0.95)	0.002
BMI adequate versus not adequate	0.97 (0.90–1.04)	0.33	0.91 (0.84–1.00)	0.04

RR: relative risk, CI: confidence interval, BMI: body mass index.
[a] Adjusted for age, area (not for salt-preserved foods), past history of diabetes mellitus, and all listed lifestyle factors.

where P_i is the prevalence of the subjects for each group i at baseline and HR_i and HR_{i+1} is the age-adjusted HR of group i and group i + 1, respectively.

Trend *P* was tested by assigning ordinal numbers to each group. All *P* values are two-sided, and statistical significance was determined at the $P<0.05$ level. We performed all statistical analyses with SAS software, version 9.1.

Results

During 727,162 person-years of follow up, we identified 3451 and 2125 new cases of cancer in men and women, respectively.

Table 2 gives the main effect of individual lifestyle factors on the development of cancer. Adherence to not smoking showed the largest risk reduction among the 5 lifestyle factors for both sexes, which was followed by being physically active, moderate drinking and eating minimum salt-preserved foods (men), and adequate BMI (women). Nonsignificant reduced risk was shown for adequate BMI in men and moderate drinking and eating minimum salt-preserved foods in women.

Table 3 shows the baseline characteristics of subjects according to the number of healthy lifestyles. Because the numbers of subjects who had 0 or 1 healthy behaviors were limited, especially in women (1.1%), these categories were combined as a single group. Those who adhered to many healthy lifestyles were older, ate more vegetables and fruits, had lower salt intake, and higher total energy.

Table 4 shows the RRs and 95% CIs of developing cancer according to the number of healthy lifestyles. For both men and women, a factor-dependent risk reduction was observed among healthy lifestyles and cancer development. Compared to 0–1 healthy lifestyle factors, the adjusted RRs and 95% CIs of adherence to 2, 3, 4, and 5 factors were 0.86 (0.78–0.95), 0.72 (0.65–0.80), 0.61 (0.54–0.69), and 0.57 (0.45–0.72) for men (*P* for trend<0.0001) and 0.86 (0.53–1.40), 0.73 (0.46–1.16), 0.68 (0.42–1.08), and 0.63 (0.39–1.01) for women (*P* for trend = 0.0003). The risk was reduced 14% and 9% by each one healthy lifestyle for men and women, respectively. When stratified by age, the risk reduction was more pronounced among women older than 60; 0.44 (0.21–0.94) for 5 lifestyles (*P* for trend = 0.0005), whereas the risk reduction remained marginally significant among women younger than 60; 0.78 (0.42–1.42; *P* for trend = 0.08). The results were similar after excluding early onset of cancer (data not shown). The PAF_{ALL} and PAF_{+1} was more than double for men compared to women; the corresponding values were 26.7% and 16.3% in men and 11.0% and 7.8% in women, respectively.

We conducted additional analyses that examined the effect of adherence to 4 healthy lifestyles (moderate drinking, minimum salted foods, physical activity, and appropriate BMI) and cancer incidence when subjects were restricted to current or former smokers. The risk was reduced 10% (significantly for men and not significantly for women) by each one healthy lifestyle. Similar results were also shown when stratifications by other factors were conducted.

When the weighted score approach was applied, the results were essentially similar to those observed in the simple binary method.

Discussion

In this large-scale population-based prospective study, a clear linear reduction of cancer risk was observed according to the adherence to the 5 healthy lifestyle factors; the risk was reduced 10% by each one healthy lifestyle.

Table 3
Baseline characteristics of subjects according to the number of healthy lifestyle factors among Japanese subjects (1995).

	Number of healthy lifestyle factors									
	Men					Women				
	0–1	2	3	4	5	0–1	2	3	4	5
No.	5720	11,602	12,313	6296	1033	274	2574	11,918	18,476	8342
Age, y	55.6	56.0	56.6	57.2	57.1	54.2	56.1	56.7	56.8	56.3
Cancer screening, %	42.8	40.8	40.7	40.1	42.9	32.9	44.1	46.3	48.7	49.8
Past history of diabetes mellitus, %	5.6	6.4	6.4	6.9	4.6	1.5	4.7	3.5	3.0	2.3
Vitamin supplement use, %	9.6	9.9	10.6	10.5	10.8	18.6	15.2	14.0	15.6	15.9
Total energy, kcal/d	2396.8	2234.8	2134.6	2040.2	2105.4	2112.4	2007.1	1910.9	1849.3	1851.8
Total vegetable, g/d	204.2	193.2	202.8	210.1	230.0	215.8	240.1	239.7	234.8	246.6
Green-yellow vegetable, g/d	93.2	87.3	91.6	95.9	106.7	94.4	109.7	108.7	106.5	112.4
Fruit, g/d	175.1	168.4	184.5	198.6	228.4	218.1	265.2	258.8	248.4	258.2
Salt, g/d	13.7	12.4	12.1	11.7	12.3	13.1	13.5	12.5	11.7	11.9
Behaviors related to healthy lifestyle factors										
Current smoking, %	97.0	85.2	60.7	20.8	0.0	86.9	34.5	10.1	2.7	0.0
Heavy smoking[a] %	54.6	44.9	30.5	10.7	0.0	30.7	10.8	2.8	0.9	0.0
Alcohol drinking, ≥150 g/week, %	91.6	69.2	36.8	13.6	0.0	66.4	16.7	3.9	0.9	0.0
Heavy drinking[b] %	60.6	42.7	21.8	7.9	0.0	28.8	6.5	1.2	0.3	0.0
Fish roe, g/d	4.4	2.0	1.1	0.7	0.4	5.8	5.1	2.7	1.3	0.5
MET, MET-h/d	30.6	31.5	33.3	35.4	42.9	28.9	28.9	29.6	31.9	37.4
BMI, kg/m^2	23.0	23.5	23.8	23.8	23.9	23.7	25.0	24.5	23.1	22.3

BMI: body mass index. Values are means unless otherwise stated.
[a] Current smoking with ≥20 cigarettes/d.
[b] Alcohol drinking ≥300 g/week.

Table 4
Relative risk (RR) of cancer incidence by number of healthy lifestyle factors adjusted by age, area, and past history of diabetes mellitus among Japanese subjects (1995–2006).

No. of healthy lifestyle factors	Men				Women			
	No. of events	Person-y	RR (95% CI)[a]	*P* for trend	No. of events	Person-y	RR (95% CI)	*P* for trend
0–1	662	52,510	1.0		18	2494	1.0	
2	1142	104,140	0.86 (0.78–0.95)		166	24,616	0.86 (0.53–1.40)	
3	1077	111,433	0.72 (0.65–0.80)		657	113,387	0.73 (0.46–1.16)	
4	492	57,570	0.61 (0.54–0.69)		914	173,403	0.68 (0.42–1.08)	
5	78	9768	0.57 (0.45–0.72)	<0.0001	370	77,842	0.63 (0.39–1.01)	0.0003
RR at one score			0.86 (0.83–0.88)				0.91 (0.87–0.96)	
PAF_{ALL},%			**26.7**				**11.0**	
PAF_{+1}, %			16.3				7.8	
Stratification by age								
Age 60>								
0–1	303	37,553	1.0		11	1974	1.0	
2	511	71,628	0.92 (0.79–1.06)		91	16,606	0.94 (0.50–1.76)	
3	441	73,950	0.74 (0.64–0.86)		350	73,427	0.82 (0.45–1.49)	
4	175	36,470	0.58 (0.48–0.70)		471	112,050	0.74 (0.41–1.34)	
5	28	6222	0.55 (0.37–0.81)	<0.0001	225	51,881	0.78 (0.42–1.42)	0.08
RR at one score			0.85 (0.81–0.89)				0.94 (0.88–1.01)	
PAF_{ALL}, %			**32.7**				**2.5**	
Age 60≤								
0–1	359	14,957	1.0		7	520	1.0	
2	631	32,512	0.81 (0.71–0.93)		75	8010	0.71 (0.33–1.53)	
3	636	37,483	0.71 (0.62–0.81)		307	39,960	0.58 (0.27–1.23)	
4	317	21,100	0.63 (0.54–0.74)		443	16,353	0.56 (0.26–1.18)	
5	50	3546	0.58 (0.43–0.78)	<0.0001	145	25,961	0.44 (0.21–0.94)	0.0005
RR at one score			0.87 (0.83–0.90)				0.88 (0.81–0.94)	
PAF_{ALL}, %			**23.8**				**22.9**	

RR: relative risk, CI: confidence interval, PAF: population attributable fraction. PAF_{ALL}: PAF that might be associated with lack of adherence to all the recommendations, PAF_{+1}: the preventable fraction when subjects would have adhered to one additional healthy lifestyle for the entire cohort except the healthiest group.
[a] Adjusted for age, area, and past history of diabetes mellitus.

In an EPIC-Potsdam Study with 7.8 years of follow up and 868 cases of cancer incidence, Ford et al. (2009) showed a 36% reduction in cancer incidence for those with all 4 factors of interest. Using a chronic disease risk index that was created to summarize how well an individual adhered to recommended health guidelines, Meng et al. (1999) showed that cancer incidence was doubled in the highest risk index group compared to the lowest risk index group in a prospective study in Hawaii. Studies focused on colorectal (Kirkegaard et al., 2010) and pancreatic cancers (Jiao et al., 2009) also showed a clear risk reduction by combinations of healthy lifestyles. Other studies used mortality data, and among them, based on Japanese population 40–79 years old at baseline, Tamakoshi et al. (2009) showed a clear risk reduction of all-cause mortality by 6 lifestyle factors.

In our study, as in previous studies (Haveman-Nies et al., 2002; Khaw et al., 2008; Tamakoshi et al., 2009), risk reduction remained statistically significant among the elderly. However, results among women aged ≤60 failed to reach statistical significance. There was no difference in baseline characteristics based on adherence to healthy lifestyles in both age groups (data not shown). The leading sites of cancer were breast (21.3%), colorectum (19.3%), stomach (13.1%), and lung (7.5%) for age ≤60 and colorectum (20.5%), stomach (14.8%), lung (12.4%), and breast (11.6%) for age ≥60. Lifetime exposure to estrogen influenced by early menarche, late natural menopause, not bearing children, and late first pregnancy increase the risk of breast cancer. These factors are not included in the 5 lifestyle factors and, thus, the results among women aged ≤60 might not have been as clear as those in the elderly or in men.

Study limitations and strengths

Our study has several limitations. First, by using binary data for lifestyle factors, we lost information on any dose response. However, when a weighted score approach was applied, similar results were obtained, in line with other studies reporting both results (Jiao et al., 2009; Kirkegaard et al., 2010). Furthermore, when subjects were restricted to current or former smokers, an essentially similar risk reduction pattern was observed. The current approach was used for its simple method that is conceptually easy to understand and can used in clinical practice. Second, we used only one point for exposure assessment. However, subjects with a past history of cancer, CVD, and ischemic heart disease were eliminated from the main analyses and after excluding early onset of cancer, similar results were obtained. Random measurement error may attenuate any results. Third, the external validity is not tested regarding the present findings. For example, salted fish roe was used to represent total salt-preserved foods or sodium intake; however, this may not necessarily be applied to other populations. The strengths of our study are its prospective design. We selected subjects from the general population, we had a large sample size, the response rate for the questionnaire was acceptable for studies of this type, and the number of subjects lost to follow-up was negligible. In addition, the cancer registry was of sufficient quality to reduce misclassification of the outcomes.

Although further studies are needed to seek cancer-causing factors or the involved mechanisms, the present analysis presents an appropriate way to translate analytic epidemiologic studies to primary cancer prevention by examining the combined effects of known modifiable lifestyle factors.

Conclusion

The combined lifestyle factors including not smoking, drinking moderately, consuming a minimum of salted foods, being physically active, and maintaining an appropriate BMI have considerable impact on the prevention of cancer.

Conflict of interest statement

None of the authors had a conflict of interest.

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We thank all staff members in each study area for their painstaking efforts to conduct the survey and follow-up. The authors' responsibilities were as follows: ST was principal investigator; MI conducted the study, managed the data collection; and SS helped to conduct the study, analyzed and interpreted the data, and prepared the manuscript. MI, NS, TS, and TY helped to conduct the study and interpreted the data. All authors provided critical suggestions for revision of the manuscript.

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Appendix A

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DDX39 acts as a suppressor of invasion for bladder cancer

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The object of the present study was to identify markers for predicting urinary bladder cancer progression by comparative proteome analysis of bladder cancers and paired normal mucosas. We found that DDX39 was overexpressed in four of six bladder cancers examined compared with respective control tissues. Immunohistochemical analysis using 303 bladder cancer specimens revealed that DDX39 was inversely correlated to pT stage and histological grade progression. The incidence of DDX39^{high} tumors (positive cells ≥50%) was 68.6%, 43.5%, 20.0%, and 5.3% in pTa, pT1, pTis, and ≥pT2 tumors, respectively, and 65.2%, 60.7%, and 19.6% in G1, G2, and G3 tumors, respectively. The incidence of DDX39^{high} tumors was significantly lower in pT1 and ≥pT2 compared to pTa tumors, and also significantly lower in G3 compared to G1 and G2 tumors. Follow-up analysis (n = 105) revealed that DDX39^{low} tumors (positive cells <50%) were associated with disease progression (hazard ratio 7.485; P = 0.0083). Furthermore, DDX39-knockdown bladder cancer cells increased their invasion ability compared to negative control cells. These results suggest that DDX39 is a suppressor of invasion and loss of its function predicts disease progression in bladder cancers. (*Cancer Sci* 2012; 103: 1363–1369)

Urinary bladder cancers account for approximately 54% of cancers of the urinary system (kidney, renal pelvis, ureters, bladder, and urethra).[1] Approximately 90% of all bladder cancers are urothelial carcinomas.[2] At initial presentation, up to 70% of tumors are non-muscle-invasive, whereas the remainder present with muscle-invasive disease.[3] The treatment for bladder cancer completely differs depending on stage. Generally, non-muscle-invasive bladder cancer (NMIBC) requires transurethral resection of the bladder tumor (TUR-Bt), whereas most muscle-invasive bladder cancer (MIBC) requires radical cystectomy with or without systemic chemotherapy. However, the prognosis for advanced bladder cancer is poor despite recent therapeutic advances.[4] To date, pathological data, including grade, stage, and associated carcinoma *in situ* (CIS) at initial presentation, have provided some insight into predicting the likelihood of progression of bladder cancer.[5,6] Nevertheless, the ability to predict progression remains a challenge as bladder tumors with the same stage and grade have a heterogeneous clinical outcome. This might be due to differences in molecular expression profile. Furthermore, understanding the molecular biology of bladder cancer may provide new therapeutic strategies.

Various molecules have been reported to be associated with the progression of bladder cancer. Tumor suppressor genes, such as *p53*, have been widely studied in bladder cancer, however, its predictive value in assessing the risk of disease progression remains controversial.[7–9] Ki-67 has some prognostic value for predicting recurrence, however, further studies are necessary and the marker is not yet clinically applicable.[10,11] Recently, proteome analysis has been widely used in the study of urine from bladder cancer patients to identify biomarkers.[12] Various urinary markers for the early detection of bladder cancer have been reported, but reliable urinary markers capable of predicting cancer progression have not been established. This is partly due to the fact that some of them are not expressed in bladder tissues.[13] Therefore, investigation of protein expression profiling in bladder cancer tissues will facilitate not only understanding the behavior of cancer cells but also identification of markers of progression of bladder cancer.

The purpose of the present study is to identify markers of bladder cancer progression by comparative proteome analyses of human bladder cancer and paired normal tissues using QSTAR Elite liquid chromatography with tandem mass spectrometry and iTRAQ technology.

Materials and Methods

Patients. Six pairs of snap-frozen bladder urothelial carcinomas and normal mucosa from cystectomy specimens were used for proteome analyses. The clinicopathological characteristics of the bladder carcinomas was as follows: case 1 was pT1, G3; case 2 was pTa, G3; case 3 was pT1, G3; case 4 was pT1, G2; case 5 was pT2a, G3; and case 6 was pT3, G3. Four cases (1–4) were NMIBCs and the remaining cases (5 and 6) were MIBCs. Immunohistochemical analysis was carried out on samples from 303 patients who were treated for bladder cancer by TUR-Bt or cystectomy at Osaka City University Hospital (Osaka, Japan) between 2000 and 2009. There were 248 men (81.8%) and 55 women (18.2%), and the median age was 68 years (range, 33–90 years). Among these patients, those with CIS and those who were incompletely resected and lost to follow-up were excluded from the study. The patients who were treated by total cystectomy were also excluded, because almost all of these patients had already progressed to muscle-invasive disease. One hundred and five TUR-Bt cases (between 2004 and 2007), for which full clinical data were available, were used for follow-up analysis. Pathologic staging was carried out according to the 2002 TNM classification system,[14] and grading was done according to the 1973 World Health Organization criteria for continuity of the study, as many samples were obtained before the 2004 criteria were published. The Institutional Review Board at Osaka City University Graduate School of Medicine approved the use of the specimens and clinical data in accordance with the Declaration of Helsinki and guidelines of Osaka City University Graduate School of Medicine.

Proteome analyses. Pathologic diagnoses of the six urothelial cancers and paired control tissues were confirmed before proteome analyses. Reagents, except for those specifically noted, were obtained from AB Sciex (Foster City, CA, USA). The

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specimens were homogenized and dissolved in 300 μL T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) with protease inhibitor. After brief ultrasonication, insoluble material was removed by centrifugation at 13 000*g* for 15 min at 4°C. Protein concentration of the supernatant was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein reduction, alkylation, and subsequent peptide labeling were carried out using iTRAQ Reagent Multiplex Kit (AB Sciex, Foster City, CA, USA). Samples (100 μg of each) were resuspended in 20 μL of dissolution buffer (0.5 M triethylammonium bicarbonate at pH 8.5). One microliter of denaturant (2% SDS) and 2 μL reducing reagent (50 nM tris-(2-carboxyethyl) phosphine) were added and incubated at 60°C for 60 min. Free sulfhydryl groups of cysteines were blocked with 1 μL cysteine blocking reagent (20 mM methyl methanethiosulfonate) and incubated at room temperature for 10 min. Trypsin solution (10 μg) was added to each sample and incubated at 37°C overnight. Tryptic peptides of each sample were labeled with iTRAQ tags by incubation at room temperature for 1 h. Each of the samples was then mixed in one tube and fractionated by six concentrations of KCl solutions (10, 50, 70, 100, 200, and 350 mM) using ICAT cation exchange cartridge. Supernatant was evaporated in a vacuum centrifuge. Peptides of each fraction were resuspended into 2 mL of 2% acetonitrile and desalted using Sep-Pak Light C18 cartridge (Waters, Milford, MA, USA). The supernatant was dissolved in 20 μL of 0.1% formic acid.

Proteome analysis was carried out with a DiNa-AI nano system (KYA Technologies, Tokyo, Japan) coupled to a QSTAR EliteHybrid mass spectrometer through a NanoSpray ion source. Protein identification was done with ProteinPilot 2.0 software (AB Sciex).

Immunohistochemical analysis of DDX39. Formalin-fixed, paraffin-embedded tissues of 303 patients with bladder cancer were analyzed by immunohistochemical staining. Sections (3 μm-thick) were cut and deparaffinized in xylene and rehydrated in alcohols and distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 5 min, followed by washing in PBS three times. Sections were then incubated with 1.5% goat serum in PBS for 15 min to bind non-specific antigens and then with rabbit polyclonal antibody to DDX39 (ab96621, 1:500; Abcam, Cambridge, MA, USA) at 4°C overnight. This was followed by incubation with biotinylated goat anti-rabbit IgG for 30 min and avidin–biotin peroxidase complex for 30 min at room temperature. Antigen was detected with 3,3-diaminobenzidine and counterstaining with hematoxylin.

Immunohistochemical analysis was carried out by two pathologists who were blinded to the clinical data (S.Y. and H.W.). Immunoreactivity of DDX39 was observed in nuclei of bladder tissues but not in the normal urothelium. Under a microscope at ×200 magnification on six random fields per sample, tissues with $\geq$50% cancer cells immunoreactive for DDX39 were defined as DDX39^{high}, and those with <50% cells immunoreactive for DDX39 were defined as DDX39^{low}.

Cell lines. Human bladder cancer cell lines T24, TCCSUP, and UMUC3 were purchased from ATCC (Rockville, MD, USA). All cells were maintained as monolayer cultures at 37°C and 5% CO_2. T24 was grown in McCoy's medium and TCCSUP and UMUC were grown in MEM. All media were supplemented with 10% FBS.

Western blot analyses. Whole cell lysates were collected using a cell scraper and resupended in CelLytic MT (Sigma, St Louis, MO, USA) with protease inhibitor. The amount of total protein was determined using a BCA protein assay kit (Pierce). Protein (15 μg of each) was loaded on 10% SDS–polyacrylamide gels. Proteins were transferred to a PVDF membrane and blocked with 5% skimmed milk in TBS buffer containing 0.1% Tween-20. The membrane was probed with primary antibody for DDX39 (ab50697, 1:100; Abcam) or β-actin (ab49900, 1:100 000; Abcam) for 1 h at room temparature. After washing, the membrane was incubated for 1 h at room temperature linked with HRP-conjugated secondary antibody (#sc-2004, 1:10 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were detected using the ECL Plus Western blotting system (GE Healthcare, Piscataway, NY, USA) and LAS-3000 image analysis system (Fujifilm, Tokyo, Japan).

Real-time PCR. Total RNA was extracted from cell lines using the RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined by Nanodrop (Thermo Scientific). RNA (1 μg) was used for cDNA synthesis using Advantage RT-for-PCR kit (Takara Bio, Tokyo, Japan). The real time RT-PCR assay was carried out with the Applied Biosystems 7500 Fast real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR reactions consisted of 10 μL of 2× TaqMan FAST Universal Master Mix (Applied Biosystems), 1 μL of 20× TaqMan Gene Expression Assay (Applied Biosystems), and 1 μg cDNA solution. The assay IDs used for real-time RT-PCR were as follows: DDX39, Hs00271794_m1; and GAPDH, Hs00266705_g1. The thermal cycle program was: 20 s at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. The data were then quantified using the comparative C_t method for relative gene expression compared with GAPDH as internal control.

Knockdown of DDX39. DDX39 expression was transiently knocked down using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DDX39-specific siRNAs (Silencer Select SiRNA; Cat.# s19917 and s19918) were obtained from Life Technologies (Grand Island, NY, USA). Non-targeting control siRNA (Pre-miR miRNA Precursor Starter Kit, Cat.# AM1540) was obtained from Life Technologies. T24 cells (3×10^5) were transiently transfected with 10 nM s19917, s19918, or control SiRNA in a six-well plate. After 24 h, cells were trypsinized and used in cell proliferation and cell invasion assays.

Cell proliferation assay. T24 cells (1×10^4/well) were seeded in a 96-well plate and transfected with 10 nM DDX39 siRNAs and control siRNA. After 24 h, cell proliferation was measured using a Cell Counting Kit-8 (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. The number of cells was measured with a microplate reader (Bio-Rad, Tokyo, Japan) at 450 nm.

Cell invasion assay. Invasion was assessed in a QCM cell invasion assay (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. Briefly, transfectants (2×10^5 cells) were seeded in the upper chamber, whereas the lower chamber was loaded with medium containing 10% FBS. After a 24-h incubation at 37°C, the cells that invaded the reverse side of the insert were dislodged by incubating the insert in 225 μL cell detachment buffer for 30 min at room temparature. Lysis buffer and CyQuant GR Dye mixture (75 μL each) were added in detachment buffer and the plate was incubated for 15 min in the dark. Then 200 μL of the mixture was transferred to a 96-well plate and measured with a fluorescence plate reader at 480/520 nm.

Statistical analysis. Statistical analyses were carried out with SPSS version 19 (IBM, Armonk, NY, USA). Fisher's exact test was used to evaluate the differences in incidence of DDX39 expression patterns among clinical and pathological parameters. The progression-free survival was defined as the time between the date of surgery and the last date of follow-up or date of progression in pT status. The curves were done using the Kaplan–Meier method with the log–rank test to assess statistical significance. Cox proportional hazards analysis was used to determine the relative contribution of various factors to the risk of progression. $P < 0.05$ was considered statistically significant.

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Results

Proteome analysis. We identified 493 proteins by proteomic analysis of six sample pairs. Overexpressed proteins were selected according to the criteria that the fold difference had to be >1.2.[15] Fifteen proteins were overexpressed in cancer tissues compared to adjacent normal tissues in four or more of six sample pairs (Table 1). To validate the results of the proteome analysis, immunohistochemical staining of the above proteins in 303 bladder specimens was carried out, except for those that have already been evaluated in bladder cancers (Ezrin, nucleophosmin, prothymosin alpha, S100 calcium binding protein A11, and S100 calcium binding protein P).[16–19] Actin-related protein 3 homolog B was not evaluated as no commercial antibody was available.

Results of immunohistochemistry analyses of DDX39, B-cell receptor-associated protein 31, chaperonin containing TCP1, FK506 binding protein 4, isocitrate dehydrogenase 1, keratin 19, myosin heavy chain 9 non-muscle, prolyl 4-hydroxylase beta polypeptide, and Y box binding protein 1 revealed that DDX39, but not the other proteins, was expressed in a different manner according to cancer stage and grade as described below, although all of them showed high expression levels in cancer compared to control tissues.

Expression of DDX39 in bladder cancers. The clinicopathological parameters of the patients whose tissues were used for immunohistochemical analysis are summarized in Table 2. As shown in Figure 1, nuclear staining of DDX39 was not observed in normal urothelium (Fig. 1D). Unexpectedly, expression levels of DDX39 were apparently lower in MIBCs when compared with NMIBCs. As summarized in Table 2, the incidence of DDX39^{low} cancers was 31%, 56%, 80%, and 95% in pTa, pT1, pTis, and ≥pT2 tumors, respectively. The incidence of DDX39^{low} cancers was significantly higher in ≥pT2 compared to pT1 and pTa cancers, and also significantly higher in pT1 when compared to pTa. Furthermore, the incidence of DDX39^{low} cancers was 35%, 39%, and 80% in G1, G2, and G3 tumors, respectively, and significantly higher in G3 when compared to G1 and G2 tumors.

Follow-up of patient outcomes and survival analysis. Correlation analysis of DDX39 expression level and recurrence/progression-free survival in 105 bladder cancer patients who were treated by TUR-Bt revealed that DDX39^{low} cancers showed rapid disease progression ($P = 0.0083$; Fig. 2A). Moreover, rapid disease progression was also evident in DDX39^{low} cancers when the 78 pTa cancers were analyzed from the above cases ($P = 0.0027$; Fig. 2B). No association was found with recurrence-free survival in either of the analyses (data not shown).

Univariate and multivariate analyses. Univariate and multivariate analyses of clinicopathological parameters and progression-free survival revealed that low expression of DDX39 was an independent risk factor for progression (Tables 3,4).

Expression levels of DDX39 and invasion ability of bladder cancer cells. The mRNA expression levels of DDX39 were analyzed by real-time PCR in T24, TCCSUP, and UMUC3 cells. T24 cells showed the highest expression level of DDX39 (Fig. 3A) but the lowest invasion ability among the three bladder cancer cell lines (Fig. 3B). As T24 showed an inverse relationship between the expression level of DDX39 and its invasion ability, similar to that observed in the bladder cancer

Table 2. Clinicopathological characteristics of patients ($n = 303$), stages and grades of bladder cancer, and DDX39 expression

Parameters	DDX39 high expression tumors (%)	*P*-value
Age (68 ± 10.5 years)		
<65 years ($n = 116$)	52 (44.8)	NS
≥65 years ($n = 187$)	103 (55.1)	
Gender		
Male ($n = 248$, 81.8%)	127 (51.2)	NS
Female ($n = 55$, 18.2%)	28 (50.1)	
Stage		
pTa ($n = 169$, 55.7%)	116 (68.6)	
pT1 ($n = 62$, 20.5%)	27 (43.5)	0.0005*
≥pT2 ($n = 57$, 18.8%)	3 (5.3)	<0.0001* <0.0001†
pTis ($n = 15$, 5.0%)	3 (20.0)	0.0003*
Grade		
G1 ($n = 66$, 21.8%)	43 (65.2)	
G2 ($n = 145$, 47.9%)	88 (60.7)	
G3 ($n = 92$, 30.4%)	18 (19.6)	<0.0001**,††

*Statistically significant from pTa. **Statistically significant from G1. †Statistically significant from pT1. ††Statistically significant from G2. NS, not significant.

Table 1. Upregulated proteins in cancer tissues using liquid chromatography with tandem mass spectrometry

Symbol	Case						Full name	Location
	1	2	3	4	5	6		
ACTR3B	4.200	2.359	ND	1.718	1.818	16.933	Actin-related protein 3 homolog B (yeast)	Unknown
BCAP31	2.797	2.186	ND	1.38	2.704	ND	B-cell receptor-associated protein 31	Cytoplasm
CCT4	4.008	1.561	1.650	1.296	ND	7.610	Chaperonin containing TCP1, subunit 4 (delta)	Cytoplasm
DDX39	4.685	1.858	2.116	ND	1.900	ND	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	Nucleus
EZR	4.961	2.315	1.536	1.776	3.113	4.704	Ezrin	Plasma membrane
FKBP4	19.763	3.820	2.308	ND	3.500	ND	FK506 binding protein 4	Nucleus
IDH1	4.618	3.137	1.278	1.580	2.662	ND	Isocitrate dehydrogenase 1 (NADP+), soluble	Cytoplasm
KRT19	3.101	4.917	ND	1.437	5.312	4.614	Keratin 19	Cytoplasm
MYH9	2.918	2.888	1.413	1.473	2.438	6.401	Myosin, heavy chain 9, non-muscle	Cytoplasm
NPM1	1.529	1.240	ND	ND	1.684	11.318	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	Nucleus
P4HB	3.690	1.431	1.378	ND	2.286	10.049	Prolyl 4-hydroxylase, beta polypeptide	Cytoplasm
PTMA	3.477	1.165	1.860	ND	2.102	3.062	Prothymosin, alpha	Nucleus
S100A11	8.414	1.991	1.311	1.512	5.261	9.114	S100 calcium binding protein A11	Cytoplasm
S100P	6.842	5.731	ND	2.128	8.834	3.450	S100 calcium binding protein P	Cytoplasm
YBX1	2.978	1.711	1.644	ND	1.912	ND	Y box binding protein 1	Nucleus

The numbers listed under cases 1–6 indicate the fold change of the protein expression level of cancer tissue compared to normal tissue in each case. ND, not detectable.

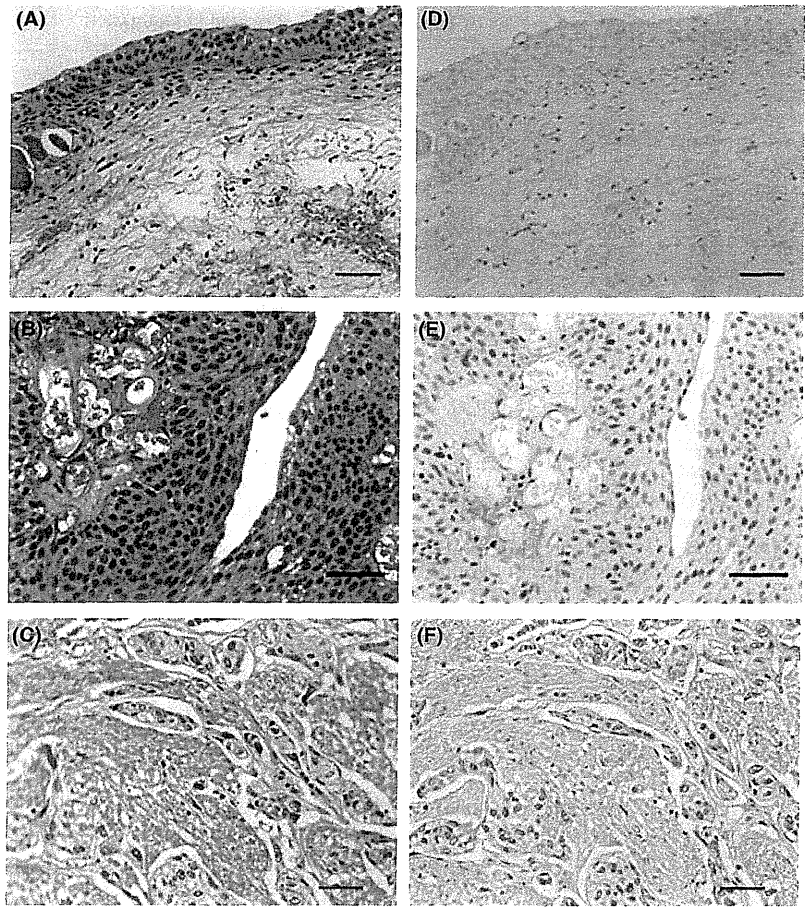

Fig. 1. Hematoxylin–eosin staining (A–C) and immunohistochemical staining (D–F) for DDX39 of normal urothelium and bladder cancers. (A,D) Normal bladder urothelium; (B,E) DDX39^{high} (positive cells ≥50%) pTa cancer; (C,F) DDX39^{low} (positive cells <50%) pT2 cancer. Bar = 50 μm.

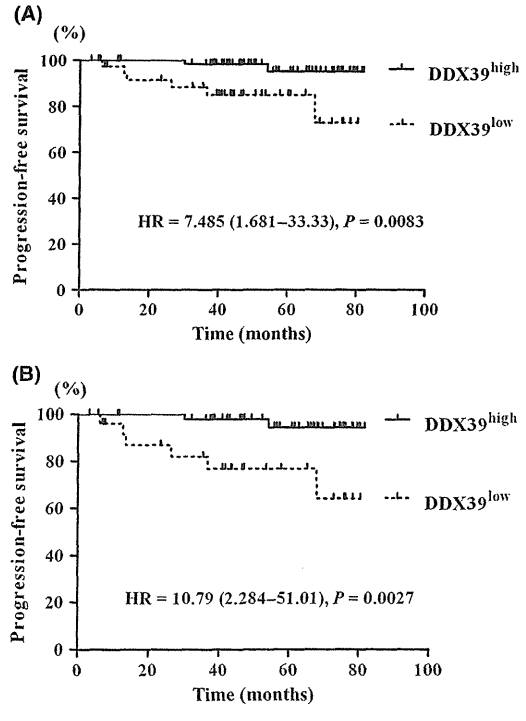

Fig. 2. DDX39^{low} cancers (positive cells <50%) showed rapid disease progression in pTa and pT1 cancers (A) (n = 105) and pTa cancers (B) (n = 78). HR, hazard ratio.

specimens analyzed above, we used T24 cells to investigate the effects of DDX39 knockdown on invasion ability.

Effect of DDX39 knockdown on cell proliferation and invasion ability of T24 cells. Western blot analysis showed a remarkable reduction in protein level of DDX39 in T24 cells transfected with si-DDX39 A (s19917) and B (s19918) compared with the negative control (Fig. 4A). Furthermore, real-time PCR analysis showed that si-DDX39 A and B reduced DDX39 mRNA expression levels by 71% and 74%, respectively, compared with the negative control (Fig. 4B). Although knockdown of DDX39 by si-DDX39 A and B had no effect on cell proliferation (Fig. 5A), T24 cells transfected with si-DDX39 A and si-DDX39 B showed 2.36- and 2.65-fold higher invasion activity, respectively, compared to the negative control (Fig. 5B).

Discussion

The results of the present study indicated that the expression level of DDX39 is significantly lower in MIBCs compared to NMIBCs. We also found that the DDX39 expression level was significantly correlated with pT stage and grade, and DDX39^{low} cancers showed rapid disease progression. Furthermore, knockdown of DDX39 increased the invasion ability of bladder cancer cells. These findings indicated that DDX39 is a suppressor of invasion and loss of its function predicts disease progression in bladder cancers. To the best of our knowledge, the present study showed for the first time the relationship between DDX39 expression and cancer cell invasion.

A member of the RNA helicases, DDX39 is involved in pre-mRNA splicing.[20] RNA helicase is thought to be required

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Table 3. Univariate analyses of various clinicopathological parameters in relation to progression-free survival of patients with bladder cancer

Patients	Progression-free survival		
Parameters	No. cases (%)	No. events	*P*-value†
Age (years)			
<65	46 (44)	2	0.2971
≥65	59 (56)	6	
Gender			
Male	84 (80)	6	0.6708
Female	21 (20)	2	
Stage			
pTa	78 (74)	8	0.1050
pT1	27 (26)	0	
Grade			
G1 + G2	90 (86)	6	0.3695
G3	15 (14)	2	
Concominant CIS			
No	98 (93)	7	0.4638
Yes	7 (7)	1	
No. tumors			
Single	76 (72)	4	0.1624
Multiple (2–7)	29 (28)	4	
Tumor size			
<3 cm	101 (96)	8	0.6123
≥3 cm	4 (4)	0	
Tumor status			
Primary	76 (72)	4	0.1830
Recurrent	29 (28)	4	
DDX39 expression			
Low (positive cells <50%)	39 (37)	6	0.0083
High (positive cells ≥50%)	66 (63)	2	

†Log–rank test. CIS, carcinoma *in situ*.

Table 4. Multivariate analyses for progression free survival

Variables	Progression-free survival	
	OR (95% CI)	*P*-value
Grade		
G1 + G2	1	–
G3	1.084 (0.140–8.420)	0.938
Carcinoma *in situ*		
–	1	–
+	2.369 (0.178–31.484)	0.514
No. tumors		
Single	1	–
Multiple	2.532 (0.570–11.250)	0.222
Prior recurrence rate		
Primary	1	–
Recurrence	1.035 (0.337–3.176)	0.952
DDX39 expression		
High	1	–
Low	7.171 (1.284–40.063)	0.025

–, normalized.

for the export of mRNA out of the nucleus, transcription, splicing, and transport of mRNA.[21] Although several other RNA helicases were reported to be dysregulated in cancer, and loss of normal function of RNA helicase could result in abnormal RNA processing,[22] little is known about the exact roles of RNA helicases in carcinogenesis. Sugiura *et al.*[23] reported that DDX39 was upregulated in lung squamous cell

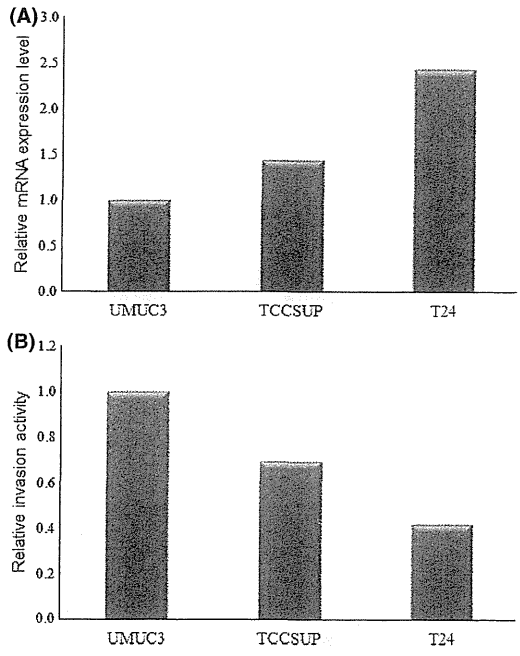

Fig. 3. mRNA expression of DDX39 (A) and cell invasion ability (B) of UMUC3, TCCSUP, and T24 bladder cancer cells. The DDX39 expression level was highest, but the invasion ability was lowest, in T24 cells compared to the other two cell lines.

cancer and promoted cancer cell growth. However, we found that DDX39 inhibits the invasion, but had no effect on proliferation, of bladder cancer cells. Although the exact role of DDX39 in bladder carcinogenesis is not known, the fact that the expression level of DDX39 is significantly higher in NMIBCs compared to MIBCs, and that DDX39 has no effect on cell proliferation and is not expressed in normal bladder mucosa, suggested that DDX39 in NMIBCs may exert a protective role against bladder cancer invasion. Furthermore, based on the finding that DDX39^{low} pTa cancers showed rapid disease progression, DDX39 might serve as a marker for NMIBCs that are likely to progress; those showing low levels of DDX39 may require more intensive therapy and closer follow-up. Further study is needed to evaluate the underlying mechanisms by which DDX39 inhibits the invasion of bladder cancer.

Matrix metalloproteinase 2 and 9 and E-cadherin were reported to be associated with bladder cancer invasion.[24,25] In the present study, the mRNA expression level of MMP2, MMP9, and E-cadherin did not change significantly by DDX39 knockdown (data not shown). These results suggested that DDX39 inhibited invasion by mechanisms independent of these proteins.

Recurrence and progression are the main problems for NMIBCs, but few reports are available identifying molecules that could predict progression. The European Association of Urology (EAU) guideline on non-muscle-invasive urothelial carcinoma of the bladder has already proven to be useful to predict recurrence and progression,[26] but its classification system is quite complicated to apply clinically. In addition, stratification based on the EAU guidelines for recurrence is not fully applicable to Japanese patients with bladder cancers.[27] In the present study, cancer stage, histological grade, concomitant CIS, number of tumors, size of tumors, and prior recurrence rate could not predict cancer progression. This might be partially due to the sample size of our patients' dataset. But the results of the present study showed that only

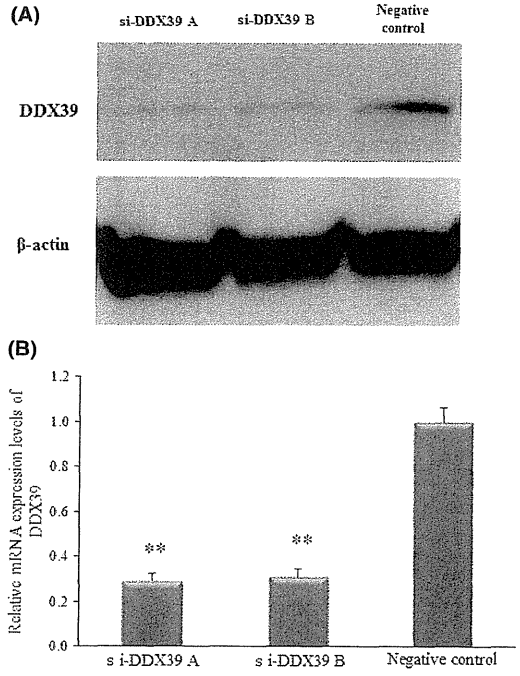

Fig. 4. Two siRNAs for DDX39 (si-DDX39 A and si-DDX39 B) down-regulated the protein (A) and mRNA expression of DDX39 (B). $^{**}P < 0.01$.

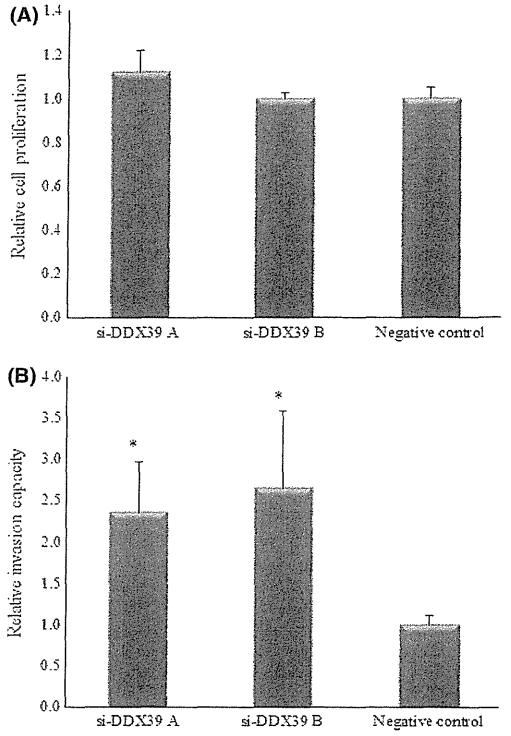

Fig. 5. siRNA knockdown effects on cell proliferation and invasion ability using T24 bladder cancer cells. (A) No significant difference in cell proliferation was found between si-DDX39 transfected cells and control cells. (B) Cells transfected with si-DDX39 A and si-DDX39 B gained significantly higher invasion ability compared to control cells. $^{*}P < 0.05$.

DDX39 had prognostic value for predicting progression of NMIBCs.

In conclusion, our results suggest that DDX39 is a suppressor of invasion and could be a useful molecular marker for predicting progression of urothelial carcinoma and a novel target for clinical therapy.

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Disclosure Statement

The authors have no conflict of interest.

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J Toxicol Pathol 2012; 25: 209–214

Original Article

Lack of Hepatocarcinogenicity of Combinations of Low Doses of 2-amino-3, 8-dimethylimidazo[4,5-*f*]quinoxaline and Diethylnitrosamine in Rats: Indication for the Existence of a Threshold for Genotoxic Carcinogens

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Abstract: The purposes of the present study were to evaluate the hepatocarcinogenicity of concurrent treatment of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and diethylnitrosamine (DEN) in rats and to determine whether no effect levels of combinations of these two different structural categories of genotoxic hepatocarcinogens exist. Two 16-week rat hepatocarcinogenesis assays were performed using a total of 790 male F344 rats. In experiment 1, we evaluated the effects of concurrent treatment of a subcarcinogenic dose of DEN on rat hepatocarcinogenesis induced by various doses of MeIQx. In experiment 2, we determined hepatocarcinogenicities of combinations of MeIQx and DEN at subcarcinogenic doses, low carcinogenic doses and high carcinogenic doses. Quantitative analyses of glutathione *S*-transferase placental form (GST-P)-positive foci, a preneoplastic lesion of the liver in rats, revealed that concurrent treatment with subcarcinogenic doses of DEN did not enhance MeIQx-induced rat hepatocarcinogenicity. We also found that concurrent treatment with combinations of subcarcinogenic doses of DEN and MeIQx was not hepatocarcinogenic, indicating that the combined effects of subcarcinogenic doses of DEN and MeIQx were neither additive nor synergistic. Moreover, concurrent treatment with low carcinogenic doses of these 2 carcinogens did not show additive or synergistic effects. Synergetic effects were observed only in rats coadministered high carcinogenic doses of the 2 carcinogens. These results demonstrate the existence of no effect levels of combinations of these 2 genotoxic hepatocarcinogens, and provide new evidence supporting our idea that there is a threshold, at least a practical threshold, that should be considered when evaluating the risk of genotoxic carcinogens. (DOI: 10.1293/tox.25.209; J Toxicol Pathol 2012; 25: 209–214)

Key words: MeIQx, DEN, concurrent treatment, carcinogenic threshold, low dose carcinogenicity

Introduction

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. It is generally assumed that genotoxic carcinogens exert a non-threshold carcinogenic effect. This concept, however, is being challenged, as advancements in the understanding of the molecular mechanisms of carcinogenesis are being made and experimental evidence continues to accumulate showing that individual genotoxic carcinogens do not exert mutagenic or carcinogenic effects at low doses[1–6]. Given the probability that humans are exposed concurrently or sequentially to trace concentrations of multiple environmental carcinogens, examination of the effects of combinations of low doses of genotoxic carcinogens is an indispensable part of cancer risk assessment.

Previous studies of carcinogenic responses in rats exposed simultaneously to multiple carcinogens indicated that additive or synergistic effects were usually evident at high exposure levels of carcinogens, but this was not always the case at low carcinogen levels (reviewed by Shirai T[7]). For example, concurrent treatment with low doses of 3 *N*-nitro-

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so compounds (genotoxic carcinogens) did not show additive effects on liver tumor development; only treatment with high doses exerted an additive effect[8]. Furthermore, concurrent treatment with low doses of 5 or 10 heterocyclic amines did not enhance development of preneoplastic lesions of the liver either additively or synergistically when given at the post-initiation stage in medium-term live bioassays (Ito test)[9–11]. These findings suggested that concurrent treatment with genotoxic carcinogens at low doses did not necessarily entail additive risk for carcinogenicity[7].

The effects of combinations of different structural categories of genotoxic carcinogens, e.g., combinations of heterocyclic amines and *N*-nitroso compounds, have not been evaluated. It also should be noted that the studies mentioned above focused mainly on the enhanced carcinogenic effects of combinations of multiple carcinogens and did not address the issue of threshold. Using various carcinogenesis models in different rat strains, we have demonstrated the existence of no effect levels for the hepatocarcinogenicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a genotoxic heterocyclic amine contained in seared fish and meat, and also for diethylnitrosamine (DEN), a genotoxic *N*-nitroso compound synthesized in the stomach through the reaction of secondary amines and nitrites in the diet[1]. The purposes of the present study were to evaluate the hepatocarcinogenicity of the concurrent treatment of MeIQx and DEN in rats and to determine whether no effect levels of combinations of these two genotoxic hepatocarcinogens exist.

Materials and Methods

Chemicals and diets

MeIQx (purity, 99.9%) was purchased from the Nard Institute (Nishinomiya, Japan), and DEN (purity>99.5%) was purchased from Sakai Research Laboratory (Fukui, Japan). Basal diet (powdered MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and diets containing MeIQx were prepared once a month by Oriental Yeast Co., Ltd., Japan.

Animals

A total of 790 male F344 rats were supplied from Charles River Laboratories Japan, Inc. (Shiga, Japan) and were used at 21 days of age. Animals were housed in polycarbonate cages (5 rats/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. Fresh diet and drinking water were supplied to the animals twice weekly. Body weights, food consumption and water intake were measured weekly.

Experimental protocols

Animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

Experiment 1 was designed to evaluate the effects of subcarcinogenic doses of DEN on MeIQx-induced rat hepatocarcinogenesis. A total of 400 rats were randomized into 12 groups and treated with test chemicals for 16 weeks as shown in the Table 1. Group 1 was the control group without administration of any of test compounds. Groups 2–6 were MeIQx-alone treatment groups that were fed diets containing MeIQx at doses of 0.01, 0.1, 1, 10, and 100 ppm, respectively. Group 7 was the DEN-alone treatment group that was administered 0.01 ppm DEN in the drinking water. Groups 8–12 were the concurrent treatment groups coadministered the above doses of MeIQx and 0.01 ppm DEN. MeIQx dose selection was based on the results of a previous low-dose MeIQx hepatocarcinogenicity study; in that study, hepatocarcinogenic effects were not observed at doses of 1 ppm MeIQx and below[12]. The dose of 0.01 ppm for DEN was the maximum noncarcinogenic dose observed in a previous low-dose DEN hepatocarcinogenicity study[12]. The treatment period of 16 weeks was the same as in our previous low-dose carcinogenicity studies[1]. All rats were killed at the end of week 16 under deep anesthesia. At necropsy, livers were excised and weighed, and then 3 slices each from the left lateral, medial, and right lateral lobes were cut and placed in 10% phosphate buffered formalin. Following fixa-

Table 1. Final Body and Liver Weights, and Intakes of Test Chemicals (Experiment 1)

Group	MeIQx (ppm)	DEN (ppm)	No. of rats	Total intake of MeIQx (mg/kg bw)	Total intake of DEN (mg/kg bw)	Body weight (g)	Liver weights	
							Absolute (g)	Relative (%)
1	—	—	40	0	0	321 ± 13	9.54 ± 0.54	2.97 ± 0.11
2	0.01	—	40	0.07	0	332 ± 15 [a]	9.79 ± 0.59	2.95 ± 0.11
3	0.1	—	40	0.71	0	338 ± 16 [a]	9.83 ± 0.63	2.91 ± 0.09
4	1	—	40	7.11	0	332 ± 14 [a]	9.76 ± 0.66	2.94 ± 0.13
5	10	—	30	71.82	0	332 ± 17 [a]	9.94 ± 0.64 [a]	2.99 ± 0.10
6	100	—	10	720.79	0	325 ± 21	11.17 ± 0.88 [a]	3.44 ± 0.08 [a]
7	—	0.01	40	0	0.095	327 ± 15	9.67 ± 0.58	2.95 ± 0.09
8	0.01	0.01	40	0.07	0.093	344 ± 18 [a,b]	9.80 ± 0.59 [a]	2.85 ± 0.08 [a,b]
9	0.1	0.01	40	0.69	0.093	340 ± 16 [a,b]	9.79 ± 0.61	2.88 ± 0.10 [a,b]
10	1	0.01	40	6.86	0.095	341 ± 21 [a,b]	9.84 ± 0.74 [a]	2.89 ± 0.12 [a,b]
11	10	0.01	30	70.42	0.095	326 ± 14	9.78 ± 0.70	2.99 ± 0.13
12	100	0.01	10	704.20	0.093	323 ± 14	11.01 ± 0.48 [a,b]	3.41 ± 0.05 [a,b]

—, 0 ppm in the diet or drinking water. [a] Significantly different from group 1. [b] Significantly different from the respective MeIQx-alone group.

tion, liver tissues were embedded in paraffin and processed for histopathological examination and immunohistochemical analysis of glutathione *S*-transferase placental form (GST-P)-positive foci, a well-established preneoplastic liver lesion in rats that can extend the range of the observable effect levels of a carcinogen and has been accepted as a reliable and sensitive end-point marker in assessment of the carcinogenic effects of environmentally relevant concentrations of carcinogens[1,7,13].

Experiment 2 was designed to evaluate the hepatocarcinogenicities of combinations of MeIQx and DEN at subcarcinogenic doses, low carcinogenic doses and high carcinogenic doses. A total of 390 male F344 rats were randomized into 16 groups and treated with test chemicals for 16 weeks as shown in Table 3. Group 1 was the control group without administration of any test compounds. Groups 2–6 were MeIQx-alone treatment groups that were administered increasing doses of MeIQx as in Experiment 1. Groups 7–11 were DEN-alone treatment groups that were administered DEN in the drinking water at doses of 0.0001, 0.001, 0.01, 0.1 and 1 ppm. The DEN dosages in this study were the same as used in previous low-dose DEN hepatocarcinogenicity studies[12]. Groups 12–16 were coadministered MeIQx and DEN as follows: subcarcinogenic dose combination groups were administered 0.01 ppm MeIQx + 0.0001 ppm DEN (group 12), 0.1 ppm MeIQx + 0.001 ppm DEN (group 13) or 1 ppm MeIQx + 0.01 ppm DEN (group 14); the low carcinogenic dose combination group was administered 10 ppm MeIQx + 1 ppm DEN (group 15); and the high carcinogenic dose combination group was administered 100 ppm MeIQx + 10 ppm DEN (group 16). At the end of week 16, all rats were necropsied, and livers were processed and analyzed as in Experiment 1.

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. GST-P-positive hepatocellular foci composed of 2 or more cells were counted under a light microscope[2,12,14,15]. Total areas of livers were measured using an IPAP color image processor (Sumica Technos, Osaka, Japan), and the number of GST-P-positive foci per square centimeter of liver tissue was calculated.

Statistical analysis

All mean values are reported as means ± SD. Statistical analyses were performed using the StatLight program (Yukms Co., Ltd., Tokyo, Japan) as described previously[2,14,16]. Briefly, homogeneity of variance was tested by the F test or Bartlett's test. Differences in mean values between the control and MeIQx- or DEN-alone treatment groups were evaluated by the 2-tailed Dunnett test when the variance was homogeneous and the 2-tailed Steel test when the variance was heterogeneous. Differences in mean values between the MeIQx- or DEN-alone groups and their respective concurrent treatment groups, and between concurrent treatment groups and the control group were evaluated by the 2-tailed Student's test when the variance was homogeneous and the 2-tailed Welch's *t* test when the variance was heterogeneous. P values less than 0.05 were considered significant.

Results

Experiment 1

The final average body and liver weights and test chemical intakes are summarized in Table 1. In the MeIQx-alone treatment groups, the final body weights of the 0.01, 0.1, 1 and 10 ppm MeIQx groups were slightly but statistically significantly higher than that of the control group (non-treatment group). Consequently, a significant increase in absolute liver weights but not the relative liver weights was observed in the 10 ppm MeIQx group compared with the control group. There was an apparent treatment-related increase in liver weight in the 100 ppm MeIQx group, as evidenced by the findings that the final body weights of this group did not significantly differ from that of the control group but both the absolute and relative liver weights were significantly increased. There were no significant differences in body weight or absolute or relative liver weight between the MeIQx-alone groups and their respective MeIQx + 0.01 ppm DEN groups. Since 0.01 ppm DEN (group 7) had no effect on body or liver weight compared with the control group, the changes in body and liver weights observed in the MeIQx + 0.01 ppm DEN groups compared with the control groups were attributed to MeIQx treatment. The intake of DEN was similar among DEN treatment groups, and the intake of MeIQx was proportional to the administered doses (Table 1). No histopathological changes were observed in the livers of any of the groups.

Total numbers of GST-P-positive foci, composed of 2 or more cells (Fig. 1), per unit area of the rat liver are shown in Table 2. In the MeIQx-alone treatment groups, the numbers of GST-P-positive foci in the livers of groups administered 0.01 ppm to 10 ppm of MeIQx did not differ from the non-treatment control value. In contrast, a significant increase was observed in the group administered 100 ppm MeIQx. As expected, 0.01 ppm DEN did not increase the number of GST-P-positive foci; in fact, the number of GST-P-positive foci in this group showed a slight but significant decrease compared with the control group. There were no significant differences in the numbers of GST-P-positive foci between the groups receiving combinations of MeIQx and 0.01 ppm DEN and their respective MeIQx-alone groups. Importantly, the numbers of GST-P-positive foci in the livers of the groups receiving combinations of 0.01 ppm to 10 ppm of MeIQx and 0.01 ppm DEN did not differ from the control value. These results indicate that the subcarcinogenic dose of 0.01 ppm DEN did not exert either additive or synergistic effects on MeIQx-induced hepatocarcinogenesis in rats.

Experiment 2

The final average body and liver weights and test chemical intakes are summarized in Table 3. MeIQx-alone

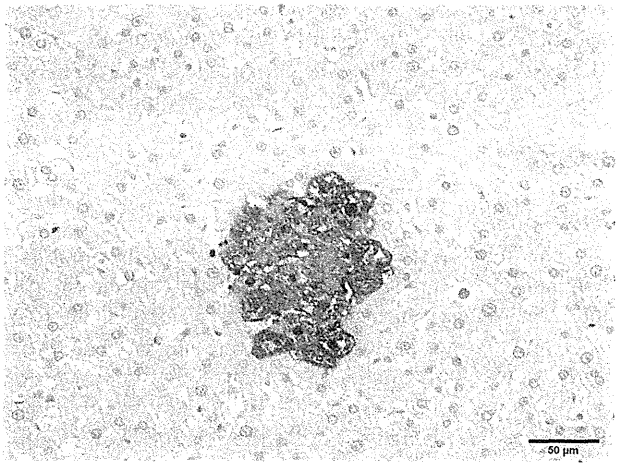

Fig. 1. GST-P-positive focus in the liver of a rat administered 100 ppm MeIQx + 1 ppm DEN (Experiment 2).

Table 2. GST-P-positive Foci in the Rat Liver (Experiment 1)

Group	MeIQx (ppm)	DEN (ppm)	No. of rats	GST-P-positive foci (no./cm^2)
1	—	—	40	0.11 ± 0.23
2	0.01	—	40	0.08 ± 0.18
3	0.1	—	40	0.10 ± 0.20
4	1	—	40	0.15 ± 0.24
5	10	—	30	0.17 ± 0.23
6	100	—	10	10.7 ± 3.97 [a]
7	—	0.01	40	0.03 ± 0.12 [a]
8	0.01	0.01	40	0.05 ± 0.14
9	0.1	0.01	40	0.10 ± 0.20
10	1	0.01	40	0.14 ± 0.26
11	10	0.01	30	0.16 ± 0.27
12	100	0.01	10	12.21 ± 7.07 [a, b]

—, 0 ppm in the diet or drinking water. [a] Significantly different from group 1. [b] Significantly different from the 100 ppm MeIQx-alone group.

Table 3. Final Body and Liver Weights, and Intakes of Test Chemicals (Experiment 2)

Group	MeIQx (ppm)	DEN (ppm)	No. of rats	Total intake of MeIQx (mg/kg bw)	Total intake of DEN (mg/kg bw)	Body weight (g)	Liver weights	
							Absolute (g)	Relative (%)
1	—	—	30	0	0	326 ± 13	8.99 ± 0.43	2.76 ± 0.10
2	0.01	—	30	0.06	0	327 ± 33	8.90 ± 0.99	2.72 ± 0.11
3	0.1	—	30	0.58	0	335 ± 15	9.30 ± 0.46 [a]	2.77 ± 0.09
4	1	—	30	5.65	0	330 ± 19	9.08 ± 0.62	2.75 ± 0.12
5	10	—	20	61.58	0	332 ± 12	9.47 ± 0.43 [a]	2.85 ± 0.11 [a]
6	100	—	10	623.29	0	326 ± 9	10.40 ± 0.48 [a]	3.19 ± 0.09 [a]
7	—	0.0001	30	0	0.001	319 ± 14	8.87 ± 0.49	2.78 ± 0.16
8	—	0.001	30	0	0.010	324 ± 15	9.20 ± 0.55	2.84 ± 0.09 [a]
9	—	0.01	30	0	0.096	315 ± 27	8.90 ± 0.74	2.83 ± 0.14
10	—	0.1	20	0	0.937	322 ± 15	9.17 ± 0.63	2.85 ± 0.15
11	—	1	10	0	10.220	317 ± 12	9.16 ± 0.55	2.89 ± 0.09 [a]
12	0.01	0.0001	30	0.06	0.001	340 ± 13 [a, b]	9.13 ± 0.46 [b]	2.69 ± 0.09 [a, b]
13	0.1	0.001	30	0.58	0.010	336 ± 13 [a, b]	9.40 ± 0.58 [a]	2.80 ± 0.12
14	1	0.01	30	5.60	0.097	334 ± 14 [a, b]	9.18 ± 0.51	2.75 ± 0.08 [b]
15	10	0.1	20	59.61	0.999	333 ± 27	9.55 ± 0.86 [a]	2.87 ± 0.09 [a]
16	100	1	10	625.68	9.655	331 ± 12 [b]	10.55 ± 0.72 [a, b]	3.19 ± 0.12 [a, b]

—, 0 ppm in the diet or drinking water. [a] Significantly different from group 1. [b] Significantly different from the respective DEN-alone group.

and DEN-alone treatments had no effect on body weights compared with the control group. No body weight suppression was observed in any combination treatment group. The final body weights of the 0.01 ppm MeIQx + 0.0001 ppm DEN, 0.1 ppm MeIQx + 0.001 ppm DEN, 1 ppm MeIQx + 0.01 ppm DEN and 100 ppm MeIQx + 1 ppm DEN treatment groups were significantly higher than their respective DEN-alone groups. Apparent treatment-related increases in both absolute and relative liver weights were observed in the groups administered 10 and 100 ppm MeIQx-alone and the groups administered 10 ppm MeIQx + 0.1 ppm DEN and 100 ppm MeIQx + 1 ppm DEN. The intake of DEN and MeIQx was proportional to the administered doses (Table 3). No histopathological changes were observed in the livers of any of the groups.

The numbers of GST-P-positive foci in rat livers are shown in Table 4. The numbers of GST-P-positive foci in the groups administered 0.01 ppm to 1 ppm MeIQx and in the groups administered 0.0001 to 0.01 ppm DEN did not differ from the non-treatment control group. Furthermore, the numbers of GST-P-positive foci in the groups administered combinations of subcarcinogenic doses of MeIQx and DEN (0.01 ppm MeIQx + 0.0001 ppm DEN, 0.1 ppm MeIQx + 0.001 ppm DEN and 1 ppm MeIQx + 0.01 ppm DEN groups) did not differ from either their respective single treatment groups or the non-treatment control group.

In the MeIQx- and DEN-alone treatment groups, significant increases in the numbers of GST-P-positive foci were observed in the groups administered 10 ppm and 100 ppm MeIQx and in the groups administered 0.1 ppm and 1 ppm DEN. However, there was no significant difference in the number of GST-P-positive foci between the 10 ppm MeIQx + 0.1 ppm DEN group and the 10 ppm MeIQx-alone or 0.1 ppm DEN-alone groups, indicating that the hepatocar-

Table 4. GST-P-positive Foci in the Rat Liver (Experiment 2)

Group	MeIQx (ppm)	DEN (ppm)	No. of rats	GST-P-positive foci (no./cm2)
1	-	—	30	0.20 ± 0.32
2	0.01	—	30	0.17 ± 0.27
3	0.1	—	30	0.24 ± 0.30
4	1	—	30	0.21 ± 0.28
5	10	—	20	0.57 ± 0.51 [a]
6	100	—	10	13.76 ± 8.78 [a]
7	—	0.0001	30	0.30 ± 0.40
8	—	0.001	30	0.29 ± 0.39
9	—	0.01	30	0.35 ± 0.48
10	—	0.1	20	0.82 ± 0.77 [a]
11	—	1	10	8.28 ± 4.27 [a]
12	0.01	0.0001	30	0.36 ± 0.48
13	0.1	0.001	30	0.17 ± 0.24
14	1	0.01	30	0.34 ± 0.51
15	10	0.1	20	0.78 ± 0.70 [a]
16	100	1	10	33.81 ± 17.46 [a,b,c]

—, 0 ppm in the diet or drinking water. [a] Significantly different from group 1. [b] Significantly different from the 100 ppm MeIQx-alone group. [c] Significantly different from the 1 ppm DEN-alone group.

cinogenicity of a combination of low carcinogenic doses of these two carcinogens was not enhanced additively or synergistically. In contrast, the number of GST-P-positive foci in the high carcinogenic dose combination group, the 100 ppm MeIQx + 1 ppm DEN group, was synergistically increased compared with the single treatment groups.

Discussion

The present study demonstrates that concurrent treatment with subcarcinogenic doses of DEN did not enhance MeIQx-induced rat hepatocarcinogenesis. We also found that concurrent treatment with combinations of subcarcinogenic doses of DEN and MeIQx were not hepatocarcinogenic, indicating that the combined effects of subcarcinogenic doses of DEN and MeIQx were neither additive nor synergistic. Moreover, concurrent treatment with low carcinogenic doses of DEN and MeIQx did not show either additive or synergistic effects. Only in rats coadministered higher carcinogenic doses of DEN and MeIQx were synergistic effects seen. The present study provides the first experimental data on the carcinogenic effects of concurrent exposure to subcarcinogenic doses of genotoxic carcinogens belonging to different structural categories. The findings of this study are in line with previous studies on the carcinogenic effects of mixtures of chemicals[7], and provide new evidence supporting our idea that no effect levels for genotoxic carcinogens exist[1,2]. Taking into account the accumulating evidence suggesting the existence of no effect levels for genotoxic carcinogens, it is reasonable to assume that the dose-response curves of genotoxic carcinogens do not reach zero in a physiologically meaningful manner, and that threshold, at least practical threshold, doses exist for the carcinogenicity of genotoxic carcinogens.

Although several threshold mechanisms for genotoxic carcinogens have been suggested, including induction of detoxification processes, cell cycle delay, DNA repair, and apoptosis and the suppression of neoplastically transformed cells by the immune system[3,17–19], the exact mechanisms are anticipated to be complicated and remain to be elucidated. This is especially true for combinations of multiple genotoxic carcinogens. We recently found that induction of $p21^{Cip/WAF1}$ and DNA repair enzymes such as AP endonuclease-1 (APE1) and GADD45 are at least partially responsible for the observed noncarcinogenic effect of low doses of 2-amino-3-methylimidazo[4,5-*f*]quinolone, a genotoxic heterocyclic amine, in rat livers[2]. Further studies are needed to clarify whether similar mechanisms contribute to the noncarcinogenic effect of low doses of MeIQx and/or DEN by evaluating the expression of genes involved in cell proliferation, DNA repair, metabolic activation and apoptosis.

Although combinations of carcinogens are generally considered to act in an additive or synergistic way with respect to cancer risk, recently it has been shown that carcinogens may interact in a non-syncarcinogenic way and, indeed, sometimes interact in an anticarcinogenic way, especially if they do not have a similar mode of carcinogenic action[7,20,21]. As reviewed by Ruediger H[21], interactive mechanisms by which one carcinogen may antagonize or attenuate the carcinogenic action of another carcinogen include inhibition of metabolic activation of procarcinogens, induction of metabolic inactivation, slowing down of the cell cycle via the p53 pathway, interference with the generation of DNA-adducts, and induction of apoptosis. The fact that concurrent treatment with low carcinogenic doses of MeIQx and DEN did not show additive or synergistic effects while combinations of the 2 carcinogens showed synergistic effects at higher carcinogenic doses suggests that combinations of MeIQx and DEN do not exert carcinogenicity in a simple dose-dependent manner and also indicates the possibility of antagonistic interaction between these 2 carcinogens when administered at low doses. Further research to clarify the interactive mechanisms mentioned above will not only facilitate the understanding of the carcinogenicity of combinations of MeIQx and DEN, but will also expand the understanding of threshold mechanisms.

In conclusion, the present study demonstrated that concurrent treatment with noncarcinogenic doses of DEN and MeIQx did not exert hepatocarcinogenicity in rats and that their combined effects were neither additive nor synergistic. These findings further support our idea that there is a threshold, at least a practical threshold, that should be considered when evaluating the risk of genotoxic carcinogens. Dose-response relationships for low dose genotoxic carcinogens, especially for mixtures of carcinogens, are still controversial in the field of carcinogen risk assessment. Further accumulation of data, especially mechanistic data, should be promoted to facilitate not only our understanding of the carcinogenic effects of low doses of genotoxic carcinogens, but also to establish accurate means of risk assessment.