Table 3
SCEs induced by river water concentrates in CHL cells in the presence of S9 mix.

Sample	Sampling date	Dose (ml/ml)[a]	SCEs per metaphase		
			MI (%)[b]	Mean ± S.D.	Range
Concentrate No. 1	14 September 2006	6.25	2.4	10.12 ± 3.50	3–18
		12.5	2.9	12.12 ± 3.94**	5–22
		18.75	2.5	16.16 ± 6.08**	6–28
		25	1.2	16.12 ± 6.35**	4–31
Concentrate No. 2	19 December 2006	6.25	3.2	12.44 ± 5.08**	5–25
		12.5	2.2	18.72 ± 5.95**	9–35
		18.75	1.5	20.84 ± 5.08**	13–37
		25	0	Toxic	Toxic
Concentrate No. 3	29 March 2007	6.25	2.8	10.64 ± 2.90	6–16
		12.5	2.4	14.00 ± 5.42**	6–28
		18.75	2.6	17.44 ± 6.87**	9–38
		25	2.1	16.36 ± 5.18**	10–29

SCE frequency for Control (DMSO) was 8.68 ± 4.28 (mean ± S.D.). SCE frequency for positive control: APNH (0.005 μg/ml) 21.44 ± 5.04 (mean ± S.D.).
[a] Dose is expressed as ml eq of river water per 1 ml of medium.
[b] MI; mitotic index. MI (%) was calculated by counting the number of mitotic cells among 1000 round nuclei.
** Significantly different from control, $p < 0.01$.

the most pronounced frequency of SCEs, showing the same level of MeIQx, although mutagenicity with *Salmonella* TA 98 and YG1024 of 5-nitro-DCB was relatively low compared with those of PBTA congeners and MeIQx as shown in Table 2. Moreover, SCE-inducing activity of 5-nitro-DCB was 1.5–2 times higher than those of PBTA congeners and was 3 times higher than that of DCB. A ranking of the SCE-inducing potency of these compounds is the following: 5-nitro-DCB ≈ MeIQx > PBTA6 > PBTA-1 ≈ PBTA-2 > DCB.

All water concentrates from the Waka River also showed dose-related increases in SCEs between the concentration of 6.25 and 18.75 ml eq/ml of medium in CHL cells with S9 mix (Table 3). SCE-inducing activities of water concentrates were from 13 to 24 ml eq/ml and a ranking of the water concentrates for SCE induction was concentrate No. 2 > 3 > 1. Both 5-nitro-DCB and DCB were detected in all water concentrates, but amounts of DCB in the water samples were about 1000-fold or higher than those of 5-nitro-DCB. Since DCB and 5-nitro-DCB showed similar SCE-inducing activity, and the amounts of DCB in the water samples were much higher than those of 5-nitro-DCB, the contribution of DCB to SCE-induction of river water concentrates may be larger than that of 5-nitro-DCB. However, contribution ratios of SCE activities based on the concentration of DCB and 5-nitro-DCB, respectively, to the total SCE activities by the river water concentrates were <3% and <0.01%, respectively. Some unknown compounds may be affecting SCE induction of the river water.

Mutagenicity of these river water concentrates was also examined by the Ames assay using YG1024 with S9 mix (Table 4). These concentrate showed potent activities, and percent contributions of DCB, i.e., 8–20%, was much higher than those of 5-nitro-DCB, which was <1%. These high percent contributions of DCB were caused by an abundance of DCB in the river water examined in this study. In a previous study, we quantified 5-nitro-DCB and DCB in blue rayon extracts from the Waka River water and detected relatively high amounts of 5-nitro-DCB [6]. The concentration of 5-nitro-DCB in the river water likely differs on sampling days. More quantitative investigations are necessary to estimate the effect of DCB and 5-nitro-DCB to the genotoxicity of the Waka River water.

In a previous study, we reported that blue rayon extracts from the water of the Yodo River system, Japan, collected in October and December, 1991, showed SCE induction in CHL cells with S9 mix [21]. PBTA-1, PBTA-2, and PBTA-6 were detected in the water samples collected from the same river system in 1994, 1995, and 1999, respectively [10,11,13]. Besides these three PBTAs, other PBTA congeners were continually detected in the water samples collected from this river system from 1994 to 2005 [15]. From synthesis studies, PBTAs are thought to be formed from corresponding dinitrophenylazo dyes used in textile dyeing factories and released into the river system. The SCE induction by the blue rayon extracts from the Yodo River system might be due to PBTA congers.

Table 4
Mutagenicity of water concentrates from the Waka River and amounts of 5-nitro-DCB and DCB.

Sample	Mutagenicity (revertants/l)[a]	Amount (ng/l)		Contribution ratio (%)[b]	
		5-Nitro-DCB	DCB	5-Nitro-DCB	DCB
Concentrate No. 1	246,200	4.8	18,900	<1	26.1
Concentrate No. 2	374,800	19.4	18,200	<1	16.5
Concentrate No. 3	179,300	2.5	4,100	<1	7.8

[a] Mutagenicity was examined in *S.typhimurium* YG1024 with S9 mix.
[b] The mutagenic potencies of 5-nitro-DCB and DCB used to calculate the contribution ratios were 24,200 and 3400 revertants/μg, respectively [6,7].

Besides 5-nitro-DCB and DCB, three dichlorobiphenyl derivatives, i.e., 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl (ADDB), 3,3′-dichloro-4,4′-dinitrobiphenyl, and 4-amino-3,3′-dichloro-4′-nitrobiphenyl, which were mutagenic in YG1024, were detected in water samples collected from the Waka River in 2003–2004 [8,9]. These DCB derivatives are thought to be formed from DCB, like 5-nitro-DCB. DCB was positive in some *in vivo* genotoxicity assays, e.g., the chromosomal aberration test [31], the micronucleus assay [32], and the alkaline single cell gel electrophoresis assay (comet assay) [33]. Moreover, DCB is carcinogenic in mice, rats, hamsters, and dogs [34], and it has been designated a probable human carcinogen (Group 2B) by IARC [35]. PBTA-1 and PBTA-2 induced micronuclei in Chinese hamster cell line V79-NZ [36]. PBTA-6 and ADDB induced micronuclei in gill cells by i.p. injection into goldfish [37]. Furthermore, DNA damaging activity was detected for PBTA-6 and ADDB in peripheral erythrocytes of goldfish *in vivo* by the comet assay [37].

Our results indicate that various dichlorobiphenyl derivatives and PBTA congeners were detected in the water of the Waka River and the Yodo River system, respectively. Except for DCB, biological activities of these water pollutants have been evaluated mostly by the Ames assay, and data on biological effects of these compounds, including genotoxicity in mammalian cells and *in vivo*, are quite limited. To estimate risks of these compounds to aquatic biota and human health, further investigations on their biological activities to aquatic organisms and experimental animals are necessary. In addition, quantitative studies on these compounds in these rivers are important, and exposure levels of aquatic organisms and human

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to those compounds need to be determined. Because aquatic organisms inhabiting in these rivers may be exposed chronically to these genotoxic chemicals, ecological studies, including the incidence of cancer in fish and aquatic animals, are also needed.

Conflicts of interest

None.

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Isolation and Identification of a Novel Aromatic Amine Mutagen Produced by the Maillard Reaction

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To clarify the formation of mutagens in the Maillard reaction of glucose and amino acids, 20 amino acids were separately incubated with glucose in the presence or absence of hydroxyl radicals produced by the Fenton reaction. After 1 week at 37 °C and pH 7.4, the reaction mixtures of glucose and tryptophan with and without the Fenton reagent showed mutagenicity toward *Salmonella typhimurium* YG1024 in the presence of a mammalian metabolic system (S9 mix). To identify mutagens in the reaction mixture, blue rayon-adsorbed material from a mixture of glucose, tryptophan, and the Fenton reagent was separated by column chromatography using various solid and mobile phases, and one mutagen, which accounted for 18% of the total mutagenicity of the reaction mixture, was isolated. The chemical structure of the mutagen was determined to be 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one (ABAQ) on the basis of ESI mass, high-resolution APCI mass, ^{1}H NMR, ^{13}C NMR, and IR spectral analyses and chemical synthesis of the mutagen. The novel aromatic amine showed high mutagenicity toward *S. typhimurium* TA98 and YG1024 with S9 mix, inducing 857 revertants of TA98 and 6007 revertants of YG1024/μg, respectively. The mutagenicity of ABAQ was comparable to that of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, which is a mutagenic and carcinogenic hetrocyclic amine in cooked meat and fish formed through the Maillard reaction at high temperature.

Introduction

The Maillard reaction is a nonenzymatic chemical reaction between reducing sugars and amino groups to form Schiff base adducts, which rearrange to form Amadori products. In the advanced Maillard reaction, the Amadori products are degraded into reactive carbonyl species, such as deoxyglucosone and methylglyoxal, and react again with free amino groups to form chromophores, fluorophores, and so forth. The Maillard reaction in vivo has been implicated in the aging process and various diseases, including diabetes, cataracts, retinopathy, and nephropathy (*1*−*3*). Elevated tissue concentrations of reactive carbonyl species are observed under pathological conditions (*4*−*6*). Pyrraline, that is, 2-amino-6-(2-formyl-5-hydroxymethylpyrrol-1-yl)hexanoic acid, is formed via the Maillard reaction between glucose and the ε-amino group of lysine (*7*) and is thought to induce biological responses, including mutations (*8, 9*). Increased levels of pyrraline were found in plasma and urine from diabetic individuals and were also detected in the sclerosed matrix of glomeruli affected by diabetic nephropathy (*10*−*14*). Many epidemiological studies have indicated positive links between diabetes and cancer of the liver, pancreas, and others (*15*−*17*). These findings suggest that mutagenic/carcinogenic compounds, such as some reactive carbonyl species and pyrraline, are formed by the Maillard reaction in vivo and increase the risk of cancer in persons with a history of diabetes. However, little is known about the chemical structure of most mutagens formed through the Maillard reaction in vivo.

In the present study, model reactions in vitro were used to find mutagens potentially produced by the Maillard reaction in vivo. Mixtures of glucose and L-amino acids were incubated at 37 °C and pH 7.4 in the presence or absence of hydroxyl radicals produced by the Fenton reaction, because hydroxyl radicals are commonly generated in vivo, for example, during inflammation (*18, 19*). The mutagenicity of the reaction mixtures was examined with the *Salmonella* assay. The mixtures of glucose and tryptophan with and without the Fenton reagent showed obvious mutagenicity. Consequently, the mixture of glucose, tryptophan, and the Fenton reagent was separated using blue rayon and column chromatography, and one mutagenic compound was isolated. The mutagen was determined to be a novel compound, a benzoazepinoqunolinone derivative, on the basis of the consistency of spectral data and chromatographic behaviors of the mutagen and the synthesized compound.

Experimental Procedures

Chemicals. Blue rayon was purchased from Funakoshi Co. Ltd. (Tokyo, Japan). L-Form of amino acids, HPLC-grade acetonitrile, and methanol were purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). All other chemicals used were of guaranteed grade.

Reaction of Glucose and Amino Acids with or without the Fenton Reagent for Mutagenicity Assays. Glucose (0.25 mmol) and the amino acid (0.5 mmol of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine) were dissolved in 0.5 M phosphate buffer (pH 7.4, 10 mL) with or without the Fenton reagent, that is, $FeSO_4$ (0.05 mmol) and 30% H_2O_2 (0.2 mL). Each solution was incubated at 37 °C for 1 or 3 weeks. All test samples were evaporated dry and dissolved in 50% dimethyl sulfoxide (DMSO,[1] 0.1 mL) for the mutagenicity assay.

Isolation of a Mutagen from the Reaction Mixture of Glucose and Tryptophan with the Fenton Reagent. Glucose (12.5 mmol), tryptophan (25 mmol), and $FeSO_4$ (2.5 mmol) were dissolved in 0.5 M phosphate buffer (pH 7.4, 500 mL), and 30% H_2O_2 (10 mL) was added to the solution. Then, the mixture was incubated at 37 °C for 1 week. The solution was diluted with 1.5 L of distilled water and treated with blue rayon (5 g) two times. The blue rayon was washed away with water, and adsorbed materials were extracted with 800 mL of methanol:ammonia−water (50:1, v/v) solution two times, as reported (*20*). The extract was evaporated dry. Part of the residue was used for the mutagenicity assay. The rest was further purified by column chromatography. An aliquot of each fraction obtained by column chromatography was tested for mutagenicity. The mutagenicity of the blue rayon extract and the eluate from columns were examined in *Salmonella typhimurium* YG1024 in the presence of S9 mix.

The blue rayon extract was applied to a Sephadex LH-20 column (35 mm × 860 mm, GE Healthcare UK Ltd., Buckinghamshire, England) and eluted with methanol. The first fraction was eluted with 280 mL. Thereafter, each fraction was eluted with a volume of 50 mL. Major mutagenic activity was observed in the fractions eluted at 1580−1680 mL. These mutagenic fractions were collected, evaporated, and then dissolved in methanol. The material was applied to an analytical grade YMC-Pack ODS-A 303 column (5 μm particle size, 4.6 mm × 250 mm, YMC Co. Ltd., Kyoto, Japan) for HPLC with a mobile phase of 30% acetonitrile in 25 mM phosphate buffer (pH 7.4) at a flow rate of 1 mL/min. Mutagenic fractions with retention times of 25−27 min were further purified on a CAPCELL PAK C18 ODS column (5 μm particle size, 4.6 mm × 250 mm, Shiseido Co. Ltd., Tokyo). By eluting the materials with 25% acetonitrile in 25 mM Tris-HCl buffer (pH 7.4) at a flow rate of 1 mL/min, two mutagenic fractions with retention times of 25−25.5 and 30.5−33 min were observed. The purity of the mutagenic compound (compound I) in the fractions with retention times of 30.5−33 min was confirmed on a second YMC-Pack ODS-A 303 column with a mobile phase of 30% acetonitrile in 25 mM phosphate buffer (pH 7.4) at a flow rate of 1 mL/min. The elutes were monitored for absorbance at 260 nm.

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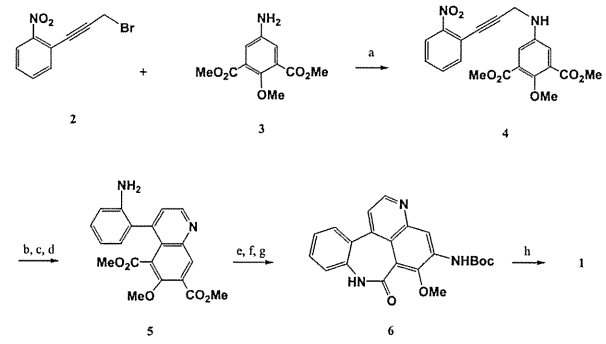
Scheme 1. Chemical Synthesis of ABAQ 1[a]



[a] Key: a, K_2CO_3; b, ICl, $NaHCO_3$; c, $Pd(PPh_3)_4$, HCO_2H; d, Pd-C/H_2; e, MsOH, *o*-dichlorobenzene; f, KOH aq; g, DPPA, *t*-BuOH; and h, BBr_3.

[1] Abbreviations: ABAQ, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one; DMSO, dimethyl sulfoxide; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

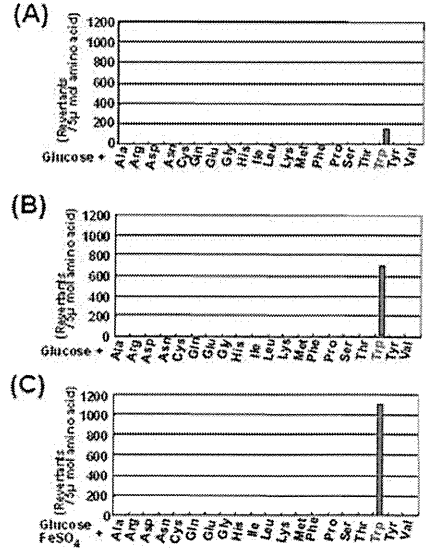


Figure 1. Mutagenicity of incubation mixtures of glucose and amino acids with or without the Fenton reagent toward *S. typhimurium* YG1024 in the presence of the S9 mix. (A) Incubation of a mixture of glucose and amino acid for 1 week, (B) incubation of a mixture of glucose and amino acid for 3 weeks, and (C) incubation of a mixture of glucose, amino acid, and the Fenton reagent for 1 week.

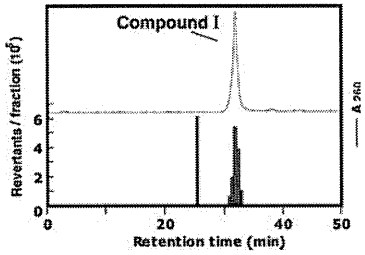


Figure 2. Purification of mutagenic compound I by HPLC. Mutagenic fractions from a YMC-Pack ODS-A 303 column with retention times of 25−27 min were purified on a CAPCELL PAK C18 ODS column. Compound I was obtained at a retention time of 32 min. The UV absorbance and mutagenicity are shown by the upper line and lower bars, respectively.

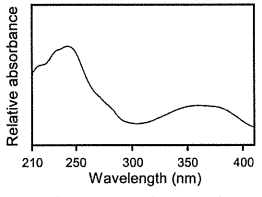


Figure 3. UV absorption spectrum of compound I, measured on the second YMC-Pack ODS-A 303 column with a photodiode array detector. The material was eluted with 30% acetonitrile in 25 mM phosphate buffer (pH 7.4).

Preparation of a Large Quantity of Compound I. In our preliminary experiment, incubation of the mixture of glucose, tryptophan, $FeSO_4$, and 30% H_2O_2 at 60 °C for 2 days with shaking enhanced the formation of compound I by about 12-fold, as compared with incubation at 37 °C for 1 week (data not shown). Glucose (62.5 mmol), tryptophan (125 mmol), and $FeSO_4$ (12.5 mmol) were dissolved in 2.5 L of 0.5 M phosphate buffer (pH 7.4), and 30% H_2O_2 (50 mL) was added to the solution. The resulting mixture was incubated at 60 °C for 2 days with shaking. In total, 90 L of mixture was incubated. Compound I in the reaction mixture was extracted with an equal amount of chloroform. The extract was then evaporated dry, and the residue was dissolved in 20 mL of methanol, filtered through a glass filter, and applied to a Sephadex LH-20 column (50 mm × 700 mm). The materials were first eluted with 460 mL of methanol, and then, methanol fractions of 40 mL were collected. The fractions at elution volumes of 2500−2820 mL, which were found to contain compound I, were combined and evaporated. The residue was dissolved in 5 mL of methanol and applied again to a Sephadex LH-20 column (30 mm × 320 mm) with methanol as a mobile phase, and fractions of 5 mL were collected after the elution of 300 mL of methanol. The fractions containing compound I, which eluted at 650−665 mL, were combined and evaporated. The residue was further purified by HPLC on a semipreparative ODS-AM 324 column (5 μm particle size, 10 mm × 300 mm, YMC Co. Ltd., Kyoto) with a mobile phase of 70% methanol at a flow rate of 2 mL/min, followed by a TSKgel CN-80Ts column (5 μm particle size, 4.6 mm × 250 mm, Tosoh Corp., Tokyo) with a mobile phase of 65% of methanol at a flow rate of 0.5 mL/min. Compound I, found in the peak fractions with retention times of 19 and 16 min on the ODS-AM 324 column and the TSKgel CN-80Ts column, respectively, was finally purified on a SUMICHIRAL OA-7100 column (5 μm particle size, 4.6 mm × 250 mm, SCAS Co. Ltd., Osaka) with a mobile phase of 50% of acetonitrile in 0.1% diethylamine−acetic acid (pH 7.4) at a flow rate of 0.5 mL/min. Compound I was isolated in the peak fraction with a retention time of 18 min. The above processes were repeated several times, and 330 μg of compound I was obtained.

Table 1. Assignments of Signals in the ^1H and ^{13}C NMR Spectra of Compound I in DMSO-d_6[a]

position	^{13}C NMR	^1H NMR
1	117.8	7.29 (1H, d, J = 4.6 Hz)
2	147.3	8.50 (1H, d, J = 4.6 Hz)
3		
3a	144.9	
4	111.9	7.20 (1H, s)
5	141.4	
6	157.4	
6a	104.5	
6b	120.3	
7	176.0	
8		10.38 (1H, s, −NH)
8a	137.0	
9	120.7	7.28 (1H, dd, J = 1.4, 7.8 Hz)
10	130.1	7.40 (1H, dt, J = 1.4, 7.8 Hz)
11	125.5	7.23 (1H, dt, J = 1.4, 7.8 Hz)
12	131.6	7.42 (1H, dd, J = 1.4, 7.8 Hz)
12a	127.7	
12b	140.6	
5-NH_2		5.78 (2H, s)

[a] Chemical shifts are expressed as ppm. s, singlet; d, doublet; dd, doublet of doublets; and dt, triplet of doublets.

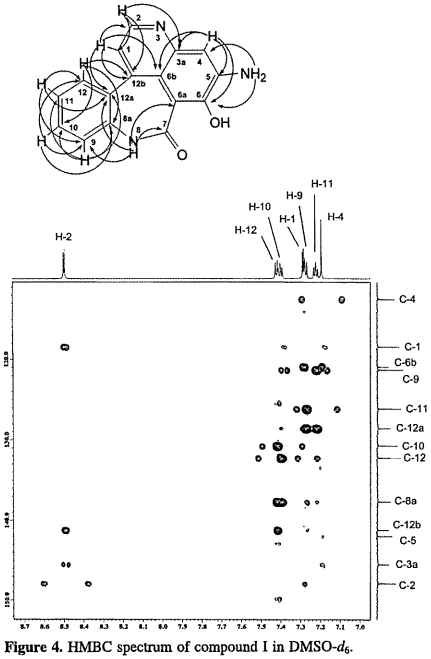


Figure 4. HMBC spectrum of compound I in DMSO-d_6.

The presence of a peak corresponding to authentic compound I was confirmed by HPLC on an analytical YMC-Pack ODS-A 303 column with a mobile phase of 30% acetonitrile in 25 mM phosphate buffer (pH 7.4) as described above.

Spectral Measurement of Compound I. ^1H NMR and ^{13}C NMR spectra were recorded with a JEOLGX-α600 or α800 instrument using microprobe FT-NMR spectrometers. The IR spectrum of microattenuated total reflection Fourier transform infrared spectroscopy was recorded with NEXUS670 and Nic-Plan with nitrogen. High-resolution mass spectrometry was performed using an API QSTAR Pulsar i equipped with a Micro-Tech HPLC system. UV absorption spectra were measured with a Tosoh PD-8020 photodiode array detector.

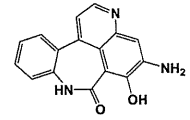


Figure 5. Chemical structure of ABAQ.

Chemical Synthesis of 5-Amino-6-hydroxy-8*H*-benzo[6,7]-azepino[5,4,3-*de*]quinolin-7-one (ABAQ) 1. 1-(3-Bromoprop-1-ynyl)-2-nitrobenzene **2** was coupled with 5-amino-2-methoxyisophthalic acid dimethyl ester **3** in the presence of potassium carbonate to afford 2-methoxy-5-[3-(2-nitrophenyl)prop-2-ynylamino]isophthalic acid dimethyl ester **4**. The coupled compound **4** was transformed to a quinoline derivative, 4-(2-aminophenyl)-6-methoxyquinoline-5,7-dicarboxylic acid dimethyl ester **5**, by using Larock's method (*21*), followed by reduction with formic acid in the presence of $Pd(PPh_3)_4$ and subsequent catalytic hydrogenation. Treatment of the quinoline derivative **5** with methanesulfuric acid provided the lactam, the methyl ester of which was saponified to carboxylic acid and further converted to *t*-butyl carbamate by Curtius rearrangement (*22*). Synchronous cleavage of the methyl ether and the *t*-Boc group in 5-*tert*-butoxycarbonylamino-6-methoxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one **6** with boron tribromide furnished ABAQ **1** (Scheme 1). Details of the preparation as well as physical properties of ABAQ and synthetic intermediates are reported elsewhere (*23*). The purity of ABAQ was above 99% on HPLC.

Mutagenicity Assay. Mutagenicity was examined by the preincubation method (*24*) using *S. typhimurum* TA98 (*25*), TA100 (*25*), YG1024 (*26*), and YG1029 (*26*) in the presence and absence of S9 mix. Samples were dissolved in DMSO, unless stated otherwise. The S9 mix contained 0.05 mL of S9 in a total volume of 0.5 mL. S9 was prepared from the liver of male Sprague−Dawley rats treated with phenobarital and β-naphtoflavone in combination.

Results

Mutagenicity of the Mixtures of Glucose and Amino Acid with or without the Fenton Reagent. Figure 1 shows the mutagenicity of mixtures of glucose and amino acids with or without the Fenton reagent toward *S. typhimurum* YG1024 in the presence of S9 mix. When the mixtures were incubated for 1 week without the Fenton reagent, only the combination of glucose and tryptophan showed mutagenicity, producing 140 revertants per 5 μmol of amino acid (Figure 1A). When the mixtures were incubated for 3 weeks, again, mutagenicity was observed only with glucose and tryptophan: 700 revertants per 5 μmol of amino acid (Figure 1B), about 5 times the level after 1 week. When the Fenton reagent was incubated with the mixtures for 1 week, glucose and tryptophan showed strong mutagenicity (Figure 1C), generating 1100 revertants per 5 μmol of amino acid, which was about eight times that after 1 week of incubation without the reagent. No mutagenicity was detected in any incubation sample without S9 mix. Solutions of each component above, that is, glucose, amino acid, or the Fenton reagent and a mixture of glucose and the Fenton reagent, which were not incubated, were not mutagenic (data not shown).

Isolation of a Mutagen from the Mixture of Glucose, Tryptophan, and the Fenton Reagent. To extract mutagens from the reaction mixture of glucose, tryptophan, and the Fenton reagent, blue rayon was used. The blue rayon extract showed mutagenicity toward *S. typhimurum* YG1024 with S9 mix, its activity accounting for 72% of the mutagenicity of the mixture. By column chromatography using Sephadex LH-20, the mutagens extracted from the blue rayon were separated into four major mutagenic fractions, fractions 13−17, fractions 19−20, fractions 22−23, and fractions 26−28, which accounted for 18, 7, 14, and 26% of the total mutagenicity of the mixture, respectively. Materials in fractions 26−28 were separated by HPLC on an analytical YMC-Pack ODS-A 303 column, and the major mutagenic fraction was observed at a retention time of 25−27 min. The mutagens in this fraction were further purified by HPLC on a CAPCELL PAK C18 ODS column. Mutagenicity was mainly recovered in the two fractions with retention times of 25−25.5 and 30.5−33 min as shown in Figure 2. These fractions accounted for 7 and 18% of the mutagenicity of the mixture, respectively. In the latter fraction (retention time of 30.5−33 min), a single UV absorption peak was observed at the same retention time. On the second YMC-Pack ODS-A 303 column, the mutagenicity of the latter fraction was confirmed to be due to a single peak, and the material was designated compound I. Three micrograms of compound I was obtained from 0.5 mmol of tryptophan and 0.25 mmol of glucose in the presence of the Fenton reagent.

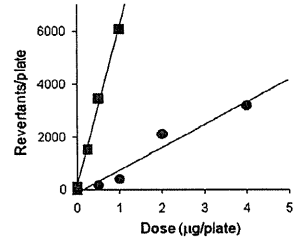
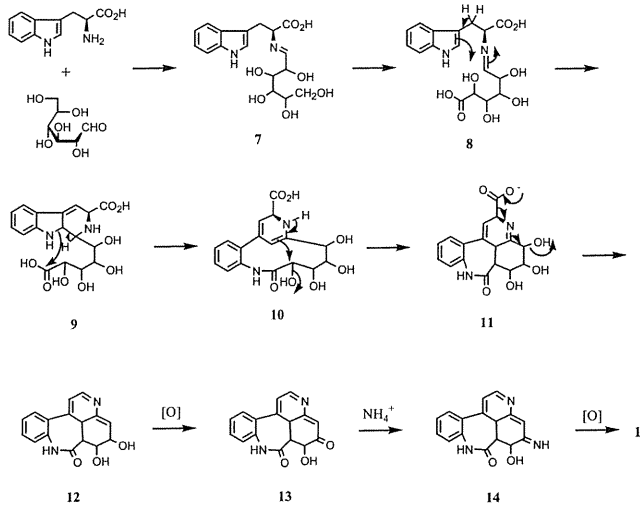


Figure 6. Mutagenicity of ABAQ toward *S. typhimurum* TA98 (●) and YG1024 (■) in the presence of S9 mix.

The UV absorption spectrum of compound I, obtained on the second YMC-Pack ODS-A 303 column with a photodiode array detector, is shown in Figure 3. Absorption maxima were found at 242 and 360 nm. With compound I isolated from the mixture described above as a marker, a large quantity of the compound was isolated from a total of 90 L of incubation mixture by column chromatography using Sephadex LH-20 and HPLC. This process was repeated several times, and 330 μg of the compound was obtained and used for various spectral analyses. The mutagenicity of compound I toward YG1024 with S9 mix was 6000 revertants/μg.

Structural Analysis of Compound I. The IR spectrum of compound I showed absorption peaks at 1641 and 1594 cm^{-1}, which suggested that an amide bond exists in the molecule, as well as a peak at 3500−3000 cm^{-1}, which suggested the presence of hydroxyl or amino groups. The mass spectrum of compound I exhibited two ion peaks, $[M + H]^+$ at *m/z* 278 in the positive mode of ESI and $[M - H]^-$ at *m/z* 276 in the negative mode. Subsequent high-resolution mass spectrometry with APCI in the negative mode indicated the molecular formula of $[M - H]^-$ to be $C_{16}H_{10}N_3O_2$ (276.0781; calculated, 276.0773). Table 1 lists chemical shifts of the proton and carbon signals in the ^1H NMR and ^{13}C NMR spectra of compound I. The ^{13}C NMR spectrum showed 16 signals only in the sp^2 carbons region. On the basis of the high-resolution mass spectrum and ^{13}C NMR spectrum, the molecular formula of compound I was confirmed to be $C_{16}H_{11}N_3O_2$. Among the 16 signals in the ^{13}C

Scheme 2. Plausible Mechanism for the Formation of ABAQ 1 from Glucose and Tryptophan



NMR spectrum, the signal at 176.0 ppm was assigned as a carbonyl carbon of an amide bond, which was observed in the IR spectrum. The ^{1}H NMR spectrum exhibited seven splitting signals at 7.20−7.42 and 8.50 ppm due to aromatic ring protons and two singlet signals at 10.38 and 5.78 ppm due to heteroatom-binding protons, which disappeared by the addition of D_2O. The doublet signal at 8.50 ppm observed in the lowest field among the carbon-binding protons was coupled with that at 7.29 ppm by a J value of 4.6 Hz. Meanwhile, a set of four signals observed at 7.23 (1H, dt, J = 1.4, 7.8 Hz), 7.28 (1H, dd, J = 1.4, 7.8 Hz), 7.40 (1H, dt, J = 1.4, 7.8 Hz), and 7.42 (1H, dd, J = 1.4, 7.8 Hz), which were correlated with one another in the ^{1}H−^{1}H COSY spectrum, indicated the presence of an *o*-substitute benzene ring. The singlet signal at 7.20 ppm did not correlate with any signals in the ^{1}H−^{1}H COSY spectrum. It is worth nothing that the proton signal at 8.50 ppm correlated with the carbon signal at 147.3 ppm in the HMQC spectrum, suggesting the presence of a pyridine or a quinoline ring in compound I. Further detailed analysis of the HMQC and the HMBC spectra revealed correlations of the proton and carbon signals (Figure 4). From the above data, the chemical structure of compound I was deduced to be ABAQ (Figure 5). The ^{1}H NMR, UV, and mass spectral data of the synthesized ABAQ were coincident with those of compound I isolated from reaction mixture from glucose, tryptophan, and the Fenton reagent. The retention times of the compound I and synthesized ABAQ by HPLC were identical. Thus, we concluded that compound I was ABAQ.

Mutagenicity of ABAQ. Synthesized ABAQ was tested for mutagenicity in *S. typhimurium* TA98, TA100, YG1024, and YG1029 with and without S9 mix. ABAQ showed potent mutagenicity toward TA98 and YG1024 in dose-dependent manner with S9 mix, and the potencies were as follows: 857 revertants of TA98 and 6007 revertants of YG1024/μg (Figure 6). ABAQ was slightly mutagenic toward TA100 (10 revertants/μg) and YG1029 (141 revertants/μg) with S9 mix. In the absence of S9 mix, ABAQ was not mutagenic in either strain.

Discussion

Some mutagenic and carcinogenic compounds in cooked foods have been reported to be formed by the Maillard reaction of reducing sugars and amino acids. A series of heterocyclic amines have been isolated as mutagens and carcinogens from cooked meat and fish, and some of them are thought to be produced through the Maillard reaction. For instance, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were suggested to be formed by the reaction of creatine with Maillard reaction products from glucose and amino acids by heating at high temperatures, such as 128 °C (*27*−*30*). The Maillard reaction also occurs at physiological temperatures. However, reports on the formation of mutagenic compounds through the Maillard reaction under physiological conditions are quite limited. In the present study, we examined the mutagenicity of reaction mixtures of glucose and amino acids held at physiological temperature and pH and found that the mixture of glucose and tryptophan kept for 1 week showed mutagenicity toward *S. typhimurium* YG1024 with S9 mix. Furthermore, under oxygen radicals- or active oxidative species-producing conditions with the Fenton reaction, the mutagenicity of the mixture of glucose and tryptophan was remarkably increased, being about eight times that after 1 week of incubation without the Fenton reagent. To identify mutagens, the reaction mixture of glucose, tryptophan, and the Fenton reagent was separated by bioassay-directed fractionation. Compound I, accounting for 18% of the mutagenicity of the mixture, was isolated and concluded to be a novel chemical, ABAQ, on the basis of the consistency of spectral data and retention times on HPLC of compound I and the synthesized compound.

ABAQ showed mutagenicity toward TA98, TA100, YG1024, and YG1029 with S9 mix, and the activities were higher in TA98 and YG1024, detectors of frameshift mutations, than TA100 and YG1029, detectors of base pair change mutations. The mutagenic potencies of ABAQ toward YG1024 and YG1029, *O*-acetyltransferase overproducing derivatives of TA98 and TA100, respectively, were higher than those toward their parent strains. These results suggest that ABAQ needs metabolism by cytochrome P450 and *O*-acetyltransferase to show mutagenicity. These characteristics were very similar to those of food-derived heterocyclic amines, reported to be formed through the Maillard reaction. The mutagenic potency of ABAQ, 857 revertants of TA98/μg and 6007 revertants of YG1024/μg, are comparable to those of PhIP, which is a mutagenic and carcinogenic heterocyclic amine (*31*).

It is possible that there might be several routes for the formation of ABAQ from glucose and tryptophan by the Maillard reaction. Scheme 2 shows one of the plausible mechanisms for the formation of ABAQ **1** from glucose and tryptophan by the Maillard reaction. First, the primary amino group of tryptophan and the aldehyde at the C-1 position of glucose were condensed to form the Schiff base **7**, the primary alcohol of which would be oxidized in the Maillard medium to carboxylic acid to afford the intermediate **8**. Next, the *ene*-reaction involving the terminal olefin of the indole, allylic proton at the β-carbon of tryptophan, and imine gave a tricyclic intermediate **9**, which was immediately converted to ε-lactam **10** accompanying deprotonation and the cleavage of a carbon−nitrogen bond. A subsequent SN2 reaction of the α-hydroxyl group of the lactam with enamine nucleophile yielded a tetracyclic compound **11** that has the basic skeleton of **1**. Decarboxylation of **11** could induce dehydration to provide the allylic alcohol **12**, which would be easily oxidized to give the α,β-unsaturated ketone **13**. The reaction of **13** with ammonium ion in the Maillard medium would form an imine intermediate **14**. Spontaneous dehydration of **14** driven by aromatic stability should furnish **1**.

In the present study, we found that a novel mutagen, ABAQ, was formed by the Maillard reaction of glucose and tryptophan in the presence and absence of hydroxyl radicals produced by the Fenton reaction. A consistent increase in blood sugar levels is a feature of diabetes, and the reaction of glucose and amino acids is thought to be enhanced in diabetic individuals. These facts suggest that ABAQ might be formed as an endogenous mutagen/carcinogen in diabetics and a population with high blood sugar levels. Further studies on the biological activities of ABAQ, such as genotocixity in vivo, and the quantification of ABAQ in biological samples from diabetic individuals are important to estimate the risk posed by ABAQ.

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Genotoxicity of nano/microparticles in *in vitro* micronuclei, *in vivo* comet and mutation assay systems

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Abstract

Background: Recently, manufactured nano/microparticles such as fullerenes (C_{60}), carbon black (CB) and ceramic fiber are being widely used because of their desirable properties in industrial, medical and cosmetic fields. However, there are few data on these particles in mammalian mutagenesis and carcinogenesis. To examine genotoxic effects by C_{60}, CB and kaolin, an *in vitro* micronuclei (MN) test was conducted with human lung cancer cell line, A549 cells. In addition, DNA damage and mutations were analyzed by *in vivo* assay systems using male C57BL/6J or *gpt* delta transgenic mice which were intratracheally instilled with single or multiple doses of 0.2 mg per animal of particles.

Results: In *in vitro* genotoxic analysis, increased MN frequencies were observed in A549 cells treated with C_{60}, CB and kaolin in a dose-dependent manner. These three nano/microparticles also induced DNA damage in the lungs of C57BL/6J mice measured by comet assay. Moreover, single or multiple instillations of C_{60} and kaolin, increased either or both of *gpt* and Spi$^-$ mutant frequencies in the lungs of *gpt* delta transgenic mice. Mutation spectra analysis showed transversions were predominant, and more than 60% of the base substitutions occurred at G:C base pairs in the *gpt* genes. The G:C to C:G transversion was commonly increased by these particle instillations.

Conclusion: Manufactured nano/microparticles, CB, C_{60} and kaolin, were shown to be genotoxic in *in vitro* and *in vivo* assay systems.

Background

Nano/microparticles are widely used because of their desirable properties in industrial, medical and cosmetic fields [1-6]. Accordingly, these particles can be released into the human environment and then can be inhaled. Most exposure to airborne nano/micromaterials occurs in the work place. Nano/microparticles can be classified into three groups: natural, anthropogenic and man-made (or artificial). The natural kind, for example, is produced during forest fires or volcanic eruptions. Anthropogenic particles are quite often a by-product of industrial activities such as welding or polishing. Diesel exhaust products, PM10 and PM2.5, well known as combustion nanoparticles, also belong to this group. The man-made group includes engineered nanomaterials [5].

Among these nano/mocroparticles, diesel exhaust particles have been well documented, in their general toxicity, mutagenicity and carcinogenicity [7-10]. In addition, asbestos, a naturally occurring nano-sized silicate mineral fiber, has been considered to be a human carcinogen [11-13]. Animal experiments and epidemiological studies have already demonstrated that pulmonary fibrosis, bronchogenic carcinomas and malignant mesotheliomas are closely associated with asbestos exposure. Another mineral fiber, titanium dioxide (TiO_2) has also been subjected to extensive research, and TiO_2 has already been shown to be carcinogenic [14]. Moreover, man-made vitreous fibres, including glass fibres, refractory ceramic fibres, and rock wool, have been sorted as carcinogens [15]. Kaolin/kaolinite is a clay mineral with the chemical composition $Al_2Si_2O_5(OH)_4$, and is used in ceramics, medicines, food additives, toothpaste and cosmetics. The largest use of kaolin is in the production of paper [3]. In 1993, W. B. Bunn 3rd *et al.* reported that increased incidences of lung tumors and mesotheliomas were observed in long-term inhalation studies of rats and hamsters treated with micro-sized refractory ceramic fibres containing kaolin as the main component [16]. However, other genotoxic and carcinogenic potentials of kaolin have not been studied *in vitro* and *in vivo*. In addition, the mechanism of cancer development by kaolin is still unclear.

On the other hand, carbon black (CB), fullerenes (C_{60}) and carbon nanotubes (CNTs) are developed as engineered nanoproducts [1,2,6,17]. Despite their highly desirable structures, their toxicity and carcinogenicity are concerns because these engineered nanoproducts are considered to be very stable and could lead to continuous inflammation when deposited in tissues. CNTs especially have received much attention from the aspect of toxicity due to their asbestos-like rod-shaped particles, and iron content [17-19]. Recently Takagi *et al.* demonstrated that multi-wall carbon nanotubes induced mesothelioma in *p53*+/- mice by a single i.p. injection [20]. In contrast, C_{60} is a spherical molecule consisting entirely of carbon atoms, and various derivatives have been reported [6,21,22]. C_{60} has widely different properties, such as scavenging of reactive oxygen species, direct interaction with biomolecules and radical formation; however, clear genotoxic and carcinogenic effects have not yet been demonstrated.

The present study aims to examine the genotoxicity/clastogenicity of widely distributed nano/microparticles such as C_{60}, CB and kaolin by an *in vitro* micronucleus test. Moreover, we analyzed the genotoxic effects of these particles by an *in vivo* comet assay and mutation assay system using *gpt* delta transgenic mice. In this mouse model, point mutations and deletions are separately analyzable by *gpt* and Spi$^-$ selections, respectively [23,24]. The mutation assay using the *gpt* delta mouse was validated and so far is widely used in the field of environmental mutagenicity.

Results

Size distribution and agglomeration state in suspensions of nano/microparticles

Figure 1 shows representative transmission electron microscope (TEM) images for the state of test materials

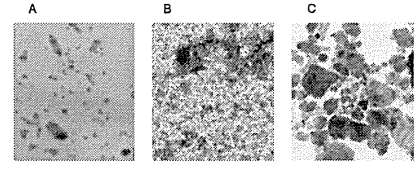

Figure 1
Representative TEM images of the presently used nano/microparticles within the suspensions. C_{60} (PanelA), CB (Panel B) and kaolin (Panel C) were suspended in saline containing 0.05% Tween 80 at a concentration of 2 mg/mL with a 10 min sonication. All images are shown at the original magnification of × 10,000.

dispersed in saline containing 0.05% Tween 80. These were commonly observed to be a mixture of well dispersed fine particles and agglomerates. C_{60} was frequently agglomerated, but fine particles were also observed either individually or within pear-shaped agglomerates. In contrast, CB was relatively well dispersed, and agglomerates were occasionally present. In the case of kaolin, low-density tabular structures with rectangular or hexagonal shape were characteristically observed. The size distribution of materials used in the present study was analyzed by dynamic light scattering (DLS). C_{60} demonstrated a wide distribution with ranges of 10.5 to 12913.9 nm, and most abundant sizes were two peaks at 234.1 ± 48.9 and 856.5 ± 119.2 nm, respectively. CB particles formed a normal distribution with ranges of 13.6 to 337.4 nm and major peak average was at around 232.0 nm. In the case of kaolin, a major peak average was 357.6 ± 199.4 nm belonging to a range of 5.1 to 4846.9 nm. Although the primary particle size of kaolin was 4.8 μm, it is likely that sonication might lead to size reduction.

In vitro *micronucleus* test

To examine the genotoxicity of particles, we analyzed the micronucleus inducing activity of C_{60}, CB and kaolin using human lung cancer cell line, A549. A six-hour treatment of 200 μg/mL CB and kaolin caused growth inhibition of 60% in A549 cells; however, C_{60} did not inhibit growth of cells at any concentrations (between 0.02 - 200 μg/mL, data not shown). As shown in Figure 2, C_{60} and kaolin particles increased the number of micronucleated cells in a dose-dependent manner. On the other hand, CB increased the number of micronucleated cells up to 2 μg/mL, and thereafter seemed to plateau. The background frequency of micronucleated cells was 0.7% to 1.0%, and the frequency rose to 10% and 5% at 200 μg/mL of C_{60} and kaolin, respectively, and 3.3% at 2 μg/mL of CB treatment. The increase of the frequency from that of the control cells was statistically significant in all particle-treated cells. C_{60} demonstrated the most strong genotoxic/clastogenic potencies among these three particles.

In vivo *genotoxicity analyzed by alkaline comet assay*

DNA damage induced by particles was evaluated using comet assay under alkaline conditions. Figure 3 shows the mean values of DNA tail moment in the lungs with or without single-particle treatment at 0.2 mg/body for 3 hr. In the case of particle exposure, DNA damage was significantly increased as compared with the vehicle control up to 2 - 3 fold, and its intensity was C_{60} > CB > kaolin. On the other hand, we examined the genotoxicity of nano/microparticles at a dose of 0.05 mg/animal. DNA damage observed in the lung of mice was almost the same as those of the vehicle control (data not shown). Moreover, we examined the effects of different exposure times for 3 and 24 hr. While DNA damages induced by CB or kaolin were

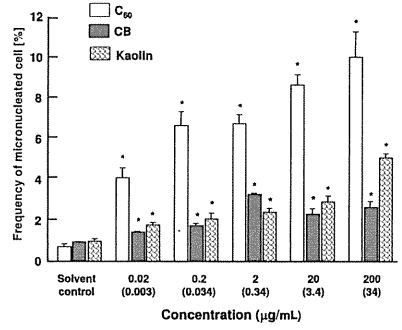

Figure 2
Frequency of micronucleated A549 cells incubated with C_{60} CB or kaolin. The values represent the mean of three experiments ± SD. An asterisk (*) represents that each frequency is significantly different ($p < 0.01$) from that of control cell in a Student's t-test. Concentrations in μg/cm² are given in parenthesis.

not changed either for 3 or 24 hr, DNA damage caused by C_{60} was decreased for 24 hr compared with 3 hr (data not shown). It seems that DNA damage repair enzymes might affect the result of comet assay.

General observations of gpt delta transgenic mice administrated with particles

Body weights of *gpt* delta mice receiving a single dose of vehicle control reached 31.1 ± 1.8 g at 12 weeks after instillation. Values for *gpt* delta mice which received a single dose of particles at 0.2 mg/body were 30.0 ± 2.4 g for C_{60}, 32.6 ± 1.1 g for CB and 30.8 ± 2.3 g for kaolin, respectively, at 12 weeks after instillation. The average consumption of diet per day per mouse was 3.6 g, with no effects from particle instillation. No body weight and diet consumption changes were also observed with multiple doses of particles. All mice used for the single dose study survived to the end of the study, although, in the case of multiple doses, one fullerene- and one kaolin-administrated mouse died within two weeks after the last instillation, probably due to respiratory disturbances.

gpt *Mutations in the lungs of* gpt *transgenic mice with particle treatment*

To determine the mutagenic effects of particles in the lungs, *gpt* delta transgenic mice were exposed to C_{60}, CB and kaolin at doses of 0.2 mg/body by single intratracheal instillation, and mutations were analyzed. Figure 3 shows the mutant frequencies (MFs) of the lungs. The background MF of lungs was 10.30 ± 0.53 × 10^{-6}. MFs in the lungs induced by C_{60} and kaolin were significantly increased by 2-fold compared with vehicle-instilled animals. CB showed increasing tendency for MF in the lungs, but not statistically significant.

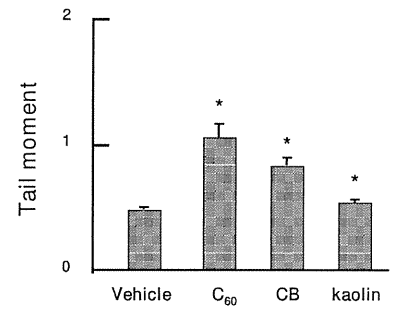

Figure 3
DNA damage in lungs of C57BL/6J mice intratracheally instilled with particles. DNA damage was measured by comet assay. Male mice were treated at a dose of 0.2 mg per animal of particles, and mice were sacrificed 3 hr after particle administrations. The values represent the mean of five animals ± SE. An asterisk (*) denotes $p < 0.01$ in a Dunnett's test after one-way ANOVA of Tail Moment of particle-treated vs. corresponding vehicle-control mice.

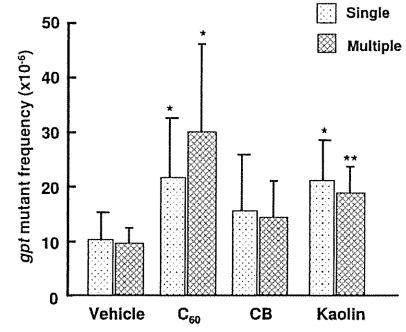

Figure 4
***gpt* MFs in the lungs of mice singly and multiply intratracheally instilled with particles.** Male mice were treated with single (0.2 mg per animal) or multiple (0.2 mg per animal × 4) doses of particles, and mice were sacrificed 12 (single) and 8 (multiple) weeks after particle administrations. Mean values ± SD are shown. An asterisk (*, **) denotes $p < 0.05$ (*) and $p < 0.01$ (**) in a Student's t-test of MF of particle-treated vs. the corresponding vehicle-control mice.

Next, we examined the mutagenic effects of consecutive exposure of particles. The *gpt* MFs in the lungs obtained from mice multiply exposed (4 times) to 0.2 mg/body each of C_{60}, CB or kaolin are shown in Figure 4. In cases of C_{60} and kaolin, MFs of the lungs were significantly higher as compared to those of control animals, and their values were 2 - 3 fold increased. In the case of CB exposure, MFs were slightly increased but not statistically significant.

To analyze the mutational characteristics induced by particles, we examined PCR and DNA sequencing analysis of 6-thioguanine (6-TG)-resistant mutants. More than 40 independent 6-TG resistant mutants derived from multiple particle instillation (0.2 mg × 4), and 25 mutants from vehicle instilled animals were identified. Classes of mutations found in the *gpt* gene are listed in Table 1. Base substitutions predominated with both particle-induced and spontaneous cases. No A:T to T:A and G:C to C:G transversions were detected in vehicle control groups, indicating that these types of mutations are rare events in the spontaneous mutations. Interestingly, G:C to C:G transversion commonly increased in all three particle treatments compared to the vehicle control. G:C to A:T transition also significantly increased in CB and kaolin instillation but not in C_{60}. In addition, the numbers of A:T to T:A transversion were slightly increased in the treatment with C_{60} and CB. Other types of mutations, including deletions and insertions, were also observed in both particle-treated and vehicle control groups, but these were of minor significance.

The distribution of spontaneous and particle-induced mutations in the *gpt* gene is shown in Figure 5. Base substitutions were spread throughout the coding region with a preference for some sites. However, clear mutational hotspots for each particle could not be seen except deletion mutations occurring at a run of 5 adenines (positions 8 to 12) and at position 244 for C_{60} treatment. The distribution of base substitutions along the *gpt* gene did not vary with the particle types. Twelve out of 200 particle-induced mutations occurred at position 64, eighteen at position 110, ten at position 115. All of the base substitutions occurring at positions 110 and 115 were G to A transitions, and at position 64 were C to T transitions, which were common among spontaneous mutants. In contrast, four to eight mutations occurred at positions 116, 143,

Table 1: Classification of gpt mutations from the lungs of control and particle multiply (0.2 mg × 4) treated mice[a]

	Control		C_{60}		CB		Kaolin	
Type of mutation in gpt	No.	%	No.	%	No.	%	No.	%
Base substitutions								
Transitions	10	40	35	41	18	45	37	50
A:T->G:C	2	8	11	13	2	5	5	7
G:C->A:T	8	32	24	28	16	40	32	43
Transversions	10	40	35	40	17	43	30	41
A:T->T:A	0	0	2	2	1	3	0	0
A:T->C:G	2	8	3	3	4	10	5	7
G:C->T:A	8	32	25	29	8	20	17	23
G:C->C:G	0	0	5	6	4	10	8	11
Deletions	4	16	12	14	4	10	6	8
Insertions	1	4	3	4	0	0	1	1
Others	0	0	1[b]	1	1[c]	3	0	0
Total	25	100	86	100	40	101	74	100

[a]Independent mutations were isolated no more than once from any individual mouse.
[b]Multiple mutation (Four base substitutions)
[c]Tandem mutation (GG->TT)

189, 320, 406 and 418 were only seen in the particle-treated mice, therefore it is suggested that these mutations can be considered as particle-induced mutations. Among these, five out of six mutations at position 406 were found in C_{60} instillation, and all mutation patterns were G to T transversions. Four out of 7 and five out of 8 at positions 189 and 418 were detected in kaolin instillation, and the majorities of the mutations were G to A and C to A, respectively. Moreover, these hotspots induced by particles occurred at G or C residues in the *gpt* gene without association for specific sequences.

Spi⁻ MFs in the lungs of gpt *transgenic mice with particle treatment*

We also measured the Spi⁻ MFs in the lungs of *gpt* delta mice instilled with multiple doses (0.2 mg × 4) of particles (Figure 6). Spi⁻ MFs of the vehicle control was 4.85 ± 2.04 × 10^{-6}, in contrast, particle-administrated groups were 4.91 ± 3.03 × 10^{-6} for C_{60}, 6.87 ± 4.06 × 10^{-6} for CB and 8.12 ± 3.32 × 10^{-6} for kaolin. As shown in Figure 6, Spi⁻ MFs in the lungs of the CB- and kaolin-treated, but not C_{60}-treated groups were increased, and in particular, the values of the kaolin-treated groups were significantly elevated up to 2-fold.

gpt Mutations in the kidneys of gpt *transgenic mice with particle treatment*

To determine the tissue distribution and specificity of particles with intratracheal instillation, *gpt* MFs of the kidney were analyzed. *gpt* MFs of the vehicle control versus particle-multiple administrated groups (0.2 mg × 4) were 1.33 ± 0.51 × 10^{-5} versus 1.67 ± 0.66 × 10^{-5} for C_{60}, 1.03 ± 0.39 × 10^{-5} for CB and 1.32 ± 0.32 × 10^{-5} for kaolin. From these observations, it is suggested that these particles did not induce mutation in the kidneys under these conditions.

Histopathological evaluation

Histopathological analyses of lung tissues of *gpt* delta mice consecutively instilled particles, C_{60}, CB and kaolin, at 0.2 mg/body per week for 4 weeks each are shown in Figure 7. Test substances-phagocytized alveolar macrophages were diffusely found in the lungs, but not in the vehicle group. Focal granulomatous formation accompanied with or without the test substance-phagocytized macrophages were also frequently observed in the lungs of particle-multiply-instilled mice. Similar findings, but a slight degree of particle accumulation and granuloma formation, were also observed in lungs of mice with particle single-instillations (data not shown). The degree of granuloma formation in the lungs of multiple C_{60}- or CB-exposed mice appeared more severe than those in multiple kaolin-exposed mice. No abnormalities were observed in the kidneys obtained from mice multiply instilled with particles (data not shown).

Discussion and conclusion

This study demonstrated the genotoxicity of nano/micro-particles widely used for industrial, cosmetic and medical fields. In *in vitro* genotoxic analysis, increased MN frequencies were observed in A549 cells treated with C_{60}, CB and kaolin in a dose-dependent manner. On the other hand, these three particles also induced DNA damage in the lungs of C57BL/6J mice measured by comet assay. Furthermore, we found that C_{60} and kaolin demonstrated mutagenicity either or both of *gpt* and Spi⁻ mutations in the *gpt* delta transgenic mice systems. The *gpt* gene MFs were significantly increased in the lungs of *gpt* delta mice with C_{60} and kaolin, but not CB administrations. A dose-dependent MF increase was observed in the lungs of C_{60}, but not kaolin treated groups. The reason is still unclear, but suggesting that the single dose of kaolin already represented the maximum response. On the other hand, kaolin demonstrated significantly increased Spi⁻ MFs; however, C_{60} showed similar values compared with the vehicle control of the lungs. Spi⁻ selection detects deletions in size more than 1 bp and 10 kb [24]; therefore, additional DNA damages involved in deletion mutations might be induced by kaolin. It, it is also suggested that C_{60} does not prefer to induce such kinds of DNA damages under these conditions. In contrast to the present study, Xu *et al.* have reported that C_{60} dramatically increases large deletion mutations in *gpt* delta transgenic mouse primary embryo fibroblast cells [25]. The observed difference of mutational signatures of C_{60} between a cell line and lung tissue might be related to differences between *in vitro* and *in vivo* assay systems in DNA damage formations, DNA repair or translesion DNA synthesis.

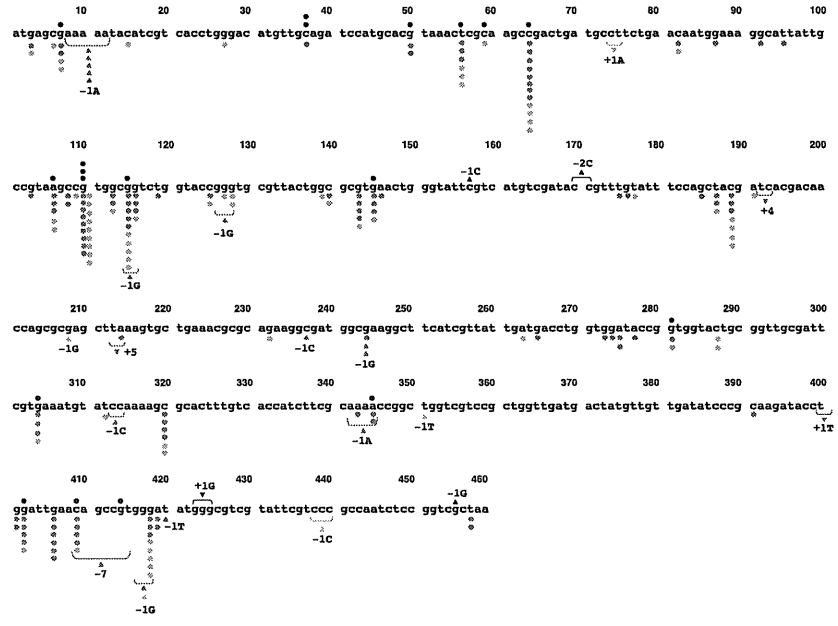

Figure 5
Spontaneous and particle-induced mutations in the coding region of the *gpt* gene. Mutations obtained from the control mice are shown above the wild type sequence, and mutations obtained from the particle-treated mutant clone are shown below the wild type sequence. The types of particles are indicated by color coding: red for C_{60}, blue for CB and sky blue for kaolin. Mutation types, base substitution, and deletion and insertion are indicated by circle, triangle, and inverted triangle, respectively.

To further elucidate the mechanisms behind the increase in mutant frequency observed in this study, we analyzed mutation spectra using a PCR-direct sequencing method. Most mutations induced by three particles in the present study, occurred at G:C base pairs (52/76, 68%). Among these, 13 G:C base pairs were located in the G or C runs. The most prominent hot spots were at base pairs 143, 189, 320, 406 and 418, and there were no significant differences in the distributions of mutation hot spots in the three particles. This may reflect the distribution of DNA damage sites caused by particles. The most prominent mutation type induced by particles was G:C to C:G transversion. Since these mutations were commonly increased regardless of the constituents of particles (i.e. C_{60} and CB were graphite and kaolin was aluminum silicate), it is suggested that mechanisms leading to the induction of such

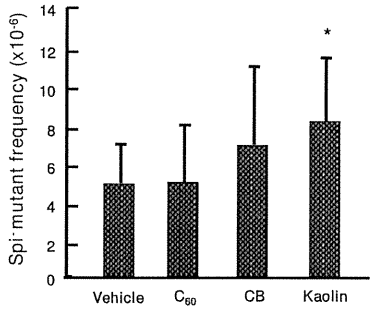

Figure 6
MFs of deletions in the lungs of *gpt* delta mice exposed to multiple doses of particles. An asterisk (*) denotes $p < 0.05$ in a Student's *t*-test of MFs of particle-treated vs. the corresponding vehicle-control mice.

kinds of mutations might be same. In general, the G:C to C:G transversion is thought to be a rare event in both spontaneous and chemically-induced mutations. However, various oxidative stresses caused by sunlight, UV radiation, hydrogen peroxide and peroxy radicals frequently induce G:C to C:G transversion in *in vitro* assay systems [26-29]. Reactive oxygen species (ROS) and DNA damage, including 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), were reported to be increased by nanoparticles, including asbestos, treatment [4,21,30-34]. The mechanism of the generation of ROS by nanoparticles is still unclear; however, these nanoparticles would be able to trigger ROS production by iron-catalysed Fenton reactions, or would be accumulated in the cells by phagocytosis, then enhance the production of ROS from macrophages and leucocytes [35,36]. In the present study, test substance-phagocytized macrophages and granulomas were frequently observed in the lungs, and the degree of the granulomas formation was partly associated with the mutagenic effect on *gpt* gene by particles. In the case of C_{60}, generation of ROS along with lipid peroxidation via electron transfer between C_{60} and other molecules has been reported [21]. The most typical lesion of oxidative damage is 8-oxo-dG which can pair with dA and leads G to T transversions [37,38] but it is not responsible for G to C transversion since dG is not incorporated opposite 8-oxodG [37,39]. Moreover, a variety of oxidative lesion products of guanine other than 8-oxodG, including imidazolone (Iz), oxazolone (Oz), spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh), have been reported

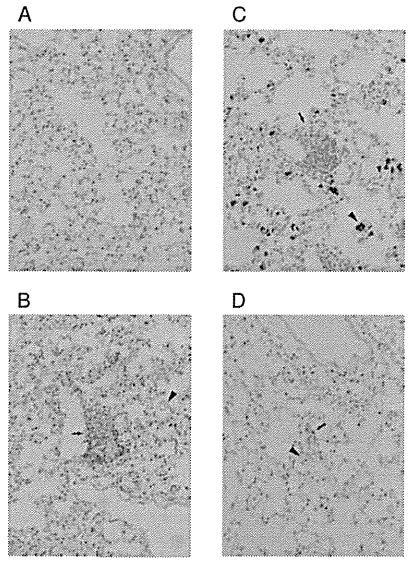

Figure 7
Microscopic findings in lungs of *gpt* delta mice intratracheally instilled with particles. Normal appearance of pulmonary parenchyma in a vehicle-control (Panel A). Pulmonary parenchyma obtained from *gpt* delta mice intratracheally instilled with four consecutive doses of 0.2 mg/mice of C_{60} (Panel B), CB (Panel C) and kaolin (Panel D). Test substance-phagocytized macrophages (arrowheads) can be observed, and granulomaous (arrows) formations are also found in lungs of particle-instilled mice. A-D; Original magnification × 40.

[39-45]. Recently, three such molecules, Oz, Sp and Gh are thought to be the key molecules causing G to C transversion using the translesion synthesis systems [43-46]. Moreover, these molecules have also been detected in bacterial cells and rat liver [47,48]. Therefore, it is suggested that G:C to C:G transversions induced by particles such as C_{60}, CB and kaolin could involve Oz, Sp and Gh formations.

In the present study, G:C to A:T transition and A:T to T:A transversion were also increased in the particle treatment. G to A transition has commonly been observed in spontaneous and chemically-induced mutants and deamination of 5-methylcytosine or alkylation of guanine might be

involved in these mutations. In contrast to G to A transition, A:T to T:A transversion is known as a rare mutation. It has been reported that the most common mutations induced by N-ethyl-N-nitrosourea in the mouse are A:T to T:A transversions [49]. However, at present, the mechanisms underlying generation of A to T transversion by particles are still unclear.

As mentioned above, we found that all three particles, C_{60}, CB and kaolin increased significant DNA damage in the lungs compared to the vehicle control using the comet assay. Comet assay under alkaline conditions is used to detect both strand breaks and DNA altering lesions such as an AP site [50]. Moreover, in the present study, treatments with C_{60}, CB and kaolin significantly increased the frequency of micronucleated A549 cells in a dose-dependent manner. However, these genotoxic/clastogenic potencies did not necessarily correspond to the mutagenicity observed in *gpt* transgenic mice.

In conclusion, we demonstrated that manufactured nano/microparticles such as C_{60}, CB and kaolin were shown to be genotoxic in both *in vitro* and *in vivo* assay systems. Moreover, it was not necessarily the case that genotoxic potency was related to particle size (C_{60} and CB are nanosized, but kaolin is micro-sized particles used in the present study.). From the prominent mutation spectra, it is suggested that oxidative DNA damage might be commonly involved in their mutagenicity. The dose of particles used in the present study seems to be extremely high compared with human exposure in the work place. However, it is likely that these materials would be deposited for a long time in tissues, same as those of asbestos fiber. Therefore, further studies of the mechanisms of genotoxicity and application routes other than trachea are needed. Moreover, exposure levels of these genotoxic particles in the working environment should be determined.

Materials and methods

Materials and chemicals

CB nanoparticles with a primary particle size of 14 nm (Printex 90) were obtained from Degussa, Dusseldorf, Germany. The surface area was 300 m^2/g (disclosed by Degussa). The CB was autoclaved at 250°C for 2 h before use. High purity (99.9%) C_{60} was purchased from Sigma-Aldrich. (St. Louis, MO, USA). The declared primary particle size of C_{60} was 0.7 nm. Kaolin, white crystal, with a primary particle size of 4.8 μm was obtained from Engelhard Corp., Iselin, NJ. C_{60}, CB and kaolin particles were suspended in saline (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) containing 0.05% of Tween 80 (Nacalai Tesque, Kyoto, Japan) by sonication for 15 - 20 min, at a concentration of 2 mg/mL. The size distributions of the presently used nano/microparticles in the suspensions were measured by dynamic light scattering (DLS) using FPAR-1000 (Otsuka electronics Co., Ltd., Osaka), and the agglomeration state was assessed by transmission electron microscope (TEM) (H-7000, Hitach, Ltd., Tokyo, Japan). The size distributions were determined with the algorithm CONTIN. For the TEM assessment, an aliquot of 5 μL was put on the nickel glid coated by hydrophilized formbar and assessed with an accelerating voltage of 75 kV.

Type I agarose, low melting point agarose, dimethylsulfoxide and Triton X-100 were bought from Sigma-Aldrich. Ethidium bromide was obtained from Merck (Darmstadt, Germany). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Micronucleus test

Human lung carcinoma A549 cells obtained from the RIKEN Cell Bank (Wako, Japan) were cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) in a 5% CO_2 atmosphere at 37°C. The cells (7×10^5 cells/dish) were seeded in plastic cell culture dishes (ϕ60 mm) one day before treatment. Particles were suspended in physiological saline containing 0.05% (v/v) Tween-80 with sonication (for 5-10 min at room temperature). One volume of the suspension was mixed with 9 volumes of the culture medium with serum (altogether 3.3 mL/dish), and then cells were treated at indicated concentrations for 6 hr. Since a long exposure (48 hr) increased the frequency of micronucleated cells in the solvent control (data not shown), we chose a 6 hr treatment. After treatment, cells were further cultured for 42 hr. Then, cells were trypsinized and counted, and centrifuged. Growth inhibition was calculated by following the formula:

Growth rate = (the number of treated cells)/(the number of non-treated cells)

Cells were resuspended in 0.075 M KCl, and incubated for 5 min. Cells were then fixed 4 times in methanol:glacial acetic acid (3:1), and washed with methanol containing 1% acetic acid. Finally, cells were resuspended in methanol containing 1% acetic acid. The cell solution was dropped onto slides and the nucleus was stained by mounting with 40 μg/mL acridine orange (Nacalai Tesque) solution and immediately observed by fluorescence microscopy using blue excitation. The number of cells with micronuclei was recorded based on observation of 1,000 interphase cells. The data of EMS and mitomycine C (MMC) for positive system controls in CHL cells under the same experimental conditions were as follows; Percentage of micronucleated cells were 9.8 ± 0.68 for EMS (1 mg/mL) and 10.3 ± 1.1 for MMC (100 n/mL), respectively.

Animals

Male C57BL/6J mice (9 weeks old) were purchased from Charles River Japan, Inc. (Atsugi, Japan) and *gpt* delta mice (9 weeks old) were obtained from Japan SLC (Shi-

zuoka, Japan), respectively. The *gpt* delta mice carry approximately 80 copies of *lambda* EG10 DNA on each chromosome 17 on a C57BL/6J background [23]. Animals were provided with food (CE-2 pellet diet, CLEA Japan, Inc., Tokyo, Japan) and tap water *ad libitum* and quarantined for one week. Mice were maintained under controlled conditions: 12-h light/dark cycle, 22 ± 2°C room temperature, and 55 ± 10% relative humidity. The experiments were conducted according to the "Guidelines for Animal Experiments in the National Cancer Center" of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

Treatment of wild type and gpt *delta transgenic mice with particles*

All particles were well sonicated and suspended in saline containing 0.05% of Tween 80. For comet assay, 5 male C57BL/6J mice were intratracheally instilled with particles using a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical, Osaka, Japan). Single doses of 0.05 or 0.2 mg per animal were employed. The control mice (n = 5) were instilled intratracheally with 0.1 mL of the solvent alone. The mice were sacrificed 3 hr after these particle administrations, and lungs were removed then used for comet assay immediately. In addition, different exposure time (24 hr) was also examined. For histological and mutation analysis, each group of 10 male *gpt* delta mice was intratracheally instilled with particles at a single dose of 0.2 mg per animal, and multiple doses of 0.2 mg per animal per week for 4 consecutive instillations, as described for comet assay. The intratracheal instillation dose of particles between 0.05 and 1 mg/mouse has been commonly used for the pulmonary inflammation and genotoxicity test [51,52]. The control mice (n = 10) were instilled intratracheally with the solvent alone. The mice were sacrificed at 22 weeks old being 12 (for single instillation) or 8 (for multiple instillations) weeks after particle administrations, respectively. Tissues, including lungs and kidneys, were removed. Lungs and kidneys obtained from 4 mice were used for histological evaluation and examined under a light microscope for any abnormalities. For histopathological evaluation, organs were fixed in 10% neutral buffered formalin, embedded in paraffin blocks and routinely processed to H&E stained sections. The remaining 6 mice were used for mutation analysis and the tissues were stored at -80°C until the DNA was isolated.

Alkaline comet assay

The alkaline comet assay was performed according to the method of Sasaki et al. [53] or Toyoizumi et al. [54] with some modification. The lungs were taken from treated mice and weighed, and lung tissue was minced and suspended with chilled homogenizing buffer, then homogenized gently using a Dounce-type homogenizer in ice. Lung cell suspension was mixed with the same volume of 1.4% low melting point agarose in PBS. The mixture was layered on the slide coated with 0.7% agarose layer, and then covered with 0.7% low melting point agarose. After slide preparation, slides were immersed in lysing solution and refrigerated at 4°C for 1 h. Each slide was then placed in alkaline electrophoresis buffer for 10 min to allow for DNA unwinding. Electrophoresis was performed at 25 V, 300 mA for 15 min at 0°C. The slides were neutralized with Tris buffer for 5 min twice, and dehydrated with 70% ethanol to fix. The cells were stained with ethidium bromide solution. Comet images were analyzed using a fluorescence microscope (magnification 200×) equipped with a CCD camera. Fifty cells were examined per mouse. The tail moment of DNA was measured using Comet Analyzer Youworks Bio Imaging Software.

gpt *and Spi⁻ mutation assays*

High-molecular-weight genomic DNA was extracted from the lungs and kidneys using a RecoverEase DNA Isolation Kit (Stratagene, La Jolla, CA) according to the instruction manual provided by the supplier. *Lambda* EG10 phages were rescued using Transpack Packaging Extract (Stratagene).

The *gpt* mutagenesis assay was performed according to previously described methods [55]. Briefly, *E. coli* YG6020 was infected with the phage and spread on M9 salt plates containing Cm and 6-TG, then incubated for 72 hr at 37°C. This enabled selection of colonies harboring a plasmid carrying the gene for chloramphenicol acetyltransferase, as well as a mutated *gpt*. Isolate exhibiting the 6-TG-resistant phenotype was cultured overnight at 37°C in LB broth containing 25 mg/mL Cm, then harvested by centrifugation (7,000 rpm, 10 min), and stored at -80°C.

The mutation spectrum of 6-TG cording sequence were performed by PCR and direct sequencing. Briefly, a 739 bp DNA fragment containing *gpt* was amplified by PCR as described previously [30,53]. Sequencing analysis was done at Takara Bio Inc. (Mie, Japan).

The Spi⁻ assay was performed as described previously [53]. The lysates of Spi⁻ mutants were obtained by infection of *E. coli* LE392 with the recovered Spi⁻ mutants. *gpt* and Spi⁻ MFs were determined in each mouse and the means ± standard deviations were calculated.

Statistical analysis

The data from micronucleus test and *gpt* and Spi⁻ mutation assay are expressed as mean ± standard deviations. The data obtained from comet assay are expressed as mean ± standard errors. The data were statistically compared with the corresponding solvent control using the Student's t-test for micronucleus and *gpt* and Spi⁻ mutation assay. To test for significant differences of tail moment in the comet assay between a group treated with materials and an untreated group, Dunnett's test after one-way ANOVA was used to evaluate the differences; p values lower than 0.05 were considered to indicate statistical significance.

Abbreviations

CB: carbon black; C_{60}: fullerenes; MN: micronuclei; CNTs: carbon nanotubes; TEM: transmission electron microscope; DLS: dynamic light scattering; MFs: mutant frequencies; 6-TG: 6-thioguanine; 8-oxo-dG: 8-oxo-7,8-dihydro-2'-deoxyguanosin; Iz: imidazolone; Oz: oxazolone; Sp: spiroiminodihydantoin; Gh: guanidinohydantoin; ROS: reactive oxygen species.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YT carried out the preparation and performance of *gpt* delta transgenic mouse experiments and drafted the manuscript. SO and MK performed *in vitro* MN tests. TK and SM performed the comet assay. TI, KH and TH performed the animal exposure and *gpt* and Spi⁻ mutation analysis. Pulmonary and renal histopathological evaluations were done by TI and AN. Analysis of size distribution and agglomeration state of particles were done by MW and NF. TN, NK, TY, TS and KW conceived and supervised the study. All authors read and approved the final manuscript.

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Analysis of an RNA adduct formed from aminophenylnorharman

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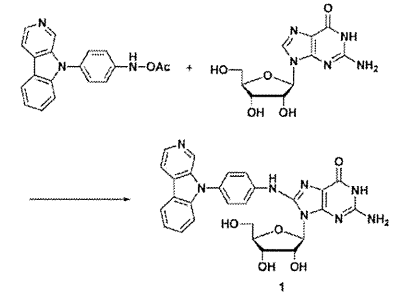
ABSTRACT

The endogenous mutagenic/carcinogenic 9- (4'-aminophenyl) -9*H*- pyrido [3,4-*b*] indole (aminophenylnorharman, APNH) is formed from norharman and aniline in the presence of cytochrome P-450s. The major APNH-DNA adduct has been reported to be 2'-deoxyguanosin-8-yl-aminophenylnorharman (dG-C8-APNH). In addition, demonstrated formation of APNH-RNA adduct and conducted a structural analysis using various spectrometric approaches. The compound produced from guanosine (Guo) and *N*-acetoxy-APNH, an ultimate mutagenic form of APNH, was concluded to be guanosin-8-yl-APNH (Guo-C8-APNH) on the basis of various spectroscopic analysis. The same adduct was found in the livers of rats administered APNH. The total adduct levels of APNH-RNA were six times higher than total APNH-DNA adducts in the same rat liver samples.



Scheme 1 A reaction mixture of *N*-acetoxy-APNH and guanosine

INTRODUCTION

Aminophenylnorharman (APNH) a product of the enzymatic reaction of norharman with aniline in the presence of S9 mix, has already been reported to be a strong mutagen/carcinogen[1-4]. Norharman and aniline abundantly exist in cigarette smoke, cooked foods and some kinds of vegetables[5,6]. Therefore, humans are exposed to both of these compounds chronically, and APNH is expected to be produced in our body. In a long term carcinogenicity experiment using experimental animals, APNH induced tumors in various tissues, including the liver and colon[3]. APNH is thought to be metabolically activated by CYP1A2 and acetyltransferase to form adducts with 2'-deoxyguanosine[7], and chemical structure of the major DNA adduct has already been reported as dG-C8-APNH, detectable in various tissues of rats and mice after a single administration of APNH[8]. In recent years, some studies have focused on RNA as a biological markers[9-11]. Similar to DNA, RNA consists of nucleobases including guanine, and would be expected to give rise to similar mutagen/carcinogen-RNA adducts. In contrast to DNA modifications by mutagens/carcinogens, which can lead to mutations, RNA modifications have generally been considered biologically meaningless. However, RNA is present in both the cytoplasmic and nuclear compartments, so it has a greater chance of reacting with the exogenous/endogenous carcinogens. Futhermore, RNA exists in a variety of forms, including tRNA, mRNA, rRNA and microRNAs, so that RNA adducts may be unique biological significance in carcinogenesis. Thus, they might offer a sensitive biomaker for exposure analysis. In the present study, we demonstrated the formation of a major APNH-RNA adduct and analyzed its chemical structure using various spectrometric approaches. In addition, we also report the generation of total APNH-RNA adducts at higher levels than total APNH-DNA lesions in the livers of rats administered APNH, as assessed by ^{32}P-postlabeling analysis[12].

RESULTS AND DISCUSSION

We first analyzed a reaction mixture of guanosine (Guo) and *N*-acetoxy-APNH (Scheme 1), an ultimate mutagenic form of APNH, by LC-ESI/MS analysis. As a result, a compound exhibiting molecular ion peak *m/z* 541 along with a fragment ion peak at *m/z* 409, consistent with loss of a ribose moiety, was found to be formed. From ^{1}H-NMR spectroscopy, its chemical structure was concluded to be guanosin-8-yl-APNH (Guo-C8-APNH) (Scheme 1). To comfirm its chemical structure, we synthesized Guo-C8-APNH via the Buchwald-Hartwig coupling reaction[13]

(Scheme 2). This product was shown to be identical to compound 1 in scheme 1 by comparison of their spectroscopic data.

Total RNA obtained from the livers of F344 rats with or without treatment of APNH was analyzed by ^{32}P-postlabeling method under adduct-intensification conditions. Adduct spot corresponding to Guo-C8-APNH were observed in the APNH-treated animals, but not in the control animals. Total adduct levels of APNH-RNA were 28±13.3 (mean±SD) adducts per 10^9 nucleotides. APNH-DNA adducts in DNA samples obtained from the same liver samples were analyzed by ^{32}P-postlabeling method under modified adduct intensification conditions. The TLC pattern was different from the case of total RNA, and their total APNH-DNA levels were 4.5±2.0 (mean±SD) adducts per 10^9 nucleotides. From these observations, it is suggested that APNH binds to both DNA and RNA *in vivo*. APNH-RNA levels were about 6 times higher than those of DNA.

CONCLUSION

We identified an APNH-RNA adduct formed by the reaction of *N*-acetoxy-APNH with Guo. The chemical structure was concluded to be Guo-C8-APNH, similar to that of dGuo-C8-APNH. Guo-C8-APNH could also be detected in rat liver after administration of APNH, suggesting that APNH can damage RNA in a manner similar to DNA *in vivo*.

We are now analyzing the function of APNH-RNA adduct using the synthesis of a Guo-C8-APNH phosphoramidite and the RNA oligonucleotide containing Guo-C8-APNH.

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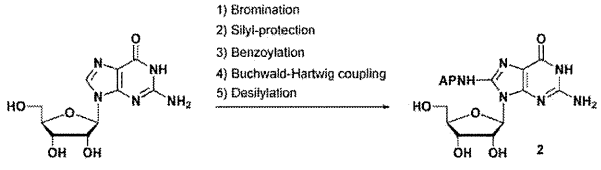

1) Br_2, water/CH_3CN; 2) DTBS(OTf)$_2$, DMF, 0 ℃ to r.t. 3) BnOH, PPh_3, DEAD/Dioxane; 4) APNH, Pd_2dba_3, xantphos, Cs_2CO_3, DMSO; 5) TBAF, THF

Scheme 2 Synthesis of Guo-C8-APNH

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Young Investigator Award Winner's Special Article

Impact of Lifestyle on Overall Cancer Risk among Japanese: The Japan Public Health Center-Based Prospective Study (JPHC Study)

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ABSTRACT

In Japan, cancer has long been recognized as a major component of the overall pattern of disease. Currently, there is a need to implement practical control measures with specific numerical targets appropriate for the Japanese population. Using data from the Japan Public Health Center-based Prospective Study, the author estimated the impact of major risk factors on overall cancer risk among a Japanese population. These risk factors included tobacco smoking, alcohol drinking, body mass index, history of diabetes, physical activity, and metabolic factors and their aggregates. The results show that tobacco smoking and heavy alcohol drinking were significantly positively associated with overall cancer risk, and that total physical activity was significantly inversely associated with the risk of cancer. Although people with a history of diabetes may be at increased risk of cancer, extreme body mass index and metabolic factors in the aggregate had little impact on overall cancer risk in the Japanese population.

Key words: cancer; risk factor; attributable fraction; Japanese; cohort study

INTRODUCTION

In Japan, cancer has been recognized as a major component of the overall pattern of disease for decades. Thus, the importance of cancer prevention through lifestyle modification is now widely acknowledged. Internationally, several studies have used epidemiologic evidence to estimate the proportion of all cancers attributable to a number of risk factors, and various international guidelines and recommendations derived from these have been promulgated.[1,2] Not surprisingly, Japanese domestic guidelines and recommendations for cancer prevention have been significantly influenced by these reports. The current need is to implement practical control measures with specific numerical targets appropriate for the Japanese population. Sufficient and reliable data derived from the Japanese population are therefore needed. Estimation of the expected effectiveness of primary prevention requires calculation of the fraction of the population incidence rate of a cancer that can be attributed to major risk factor. However, there are limited data on major risk factors and subsequent cancer risk in Japan.

We launched a large-scale, population-based, prospective study in 1990 in 11 public health center-based areas throughout Japan. The subjects were 140 420 middle-aged residents, and information was collected by using questionnaire surveys, blood samples, health screening data, and a thorough follow-up system.[3] The follow-up period is currently 10 to 15 years and a sufficient number of incident cancers has accumulated. Here, to develop a relevant epidemiological index of the impact of major risk factors—tobacco smoking, alcohol drinking, body mass index (BMI), history of diabetes mellitus (DM), physical activity, and metabolic factors and their aggregates—on overall cancer risk among the Japanese general population, we conducted cohort analyses using data from the Japan Public Health Center-based Prospective Study (JPHC study).

THE JPHC STUDY

The JPHC Study was launched in 1990 for Cohort I and in 1993 for Cohort II. Cohort I comprised 5 prefectural public health center (PHC) areas: Ninohe (Iwate Prefecture), Yokote (Akita Prefecture), Saku (Nagano Prefecture), Chubu (Okinawa Prefecture), and Katsushika (metropolitan Tokyo). Cohort II comprised 6 PHC areas: Mito (Ibaraki Prefecture), Nagaoka (Niigata Prefecture), Chuo-higashi (Kochi Prefecture), Kamigoto (Nagasaki Prefecture), Miyako (Okinawa Prefecture), and Suita (Osaka Prefecture). Details

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*The members of the study group are listed in the Appendix.

of the study design are described elsewhere.[3,4] The study population was defined as all registered Japanese inhabitants aged 40 to 59 years (for Cohort I) or 40 to 69 years (for Cohort II) at baseline. Participants were identified by resident registries maintained by local municipalities. This study was approved by the institutional review board of the National Cancer Center of Japan. In the present series of analyses, the Katsushika PHC area was excluded because data on cancer incidence were not available.

A baseline self-administered questionnaire survey was conducted in 1990 to 1994, and a 5-year follow-up questionnaire in 1995 to 1999, with a response rate of around 80%. Subjects with a history of cancer at any site were excluded from the analysis.

Subjects were followed from the starting point until the end of follow-up, which depended on the particular analysis. Residence status, including survival, was confirmed through the residential registry. Access to the resident registry is available to anyone, as mandated by the resident registration law. Among the study subjects, approximately 0.5% were lost to follow-up during the follow-up period. Information on the cause of death for deceased subjects was obtained from death certificates (provided by the Ministry of Health, Labour, and Welfare, with the permission of the Ministry of Internal Affairs and Communications), on which cause of death is classified according to the International Classification of Diseases, Tenth Revision.[5] Resident registration and death registration are required by law in Japan, and the registries are believed to be complete. Incident cancers were identified via notification from the major hospitals in the study area and by data linkage with population-based cancer registries. Death certificates were used as a supplementary information source. The site and histology of each cancer were coded using the International Classification of Diseases for Oncology, Third Edition.[6] In our cancer registry system, the proportion of cases for which information was available from death certificates only (DCO) was around 4%.

Hazard ratios (HRs) and their 95% confidence intervals (95% CIs) were used to describe the relative risk of overall cancer occurrence associated with the presence of major risk factors at the start of each study. The Cox proportional hazards model was used for the analysis, after controlling for potential confounding factors in addition to age and study area.

To express the impact of major risk factors on overall cancer occurrence in this population, the population attributable fraction (PAF) was estimated. This is the fraction of the population incidence rate of cancer that can be attributed to a particular cause—in other words, the reduction in incidence that would be expected had the population been entirely unexposed.[7] PAF was estimated as:

$$\mathrm{pd} \times \left(\mathrm{HR} - \frac{1}{\mathrm{HR}}\right)$$

where **pd** is the proportion of cases exposed to a particular risk factor. This formula is believed to have greater validity, when confounding variables are present, than the more common formula:

$$\frac{\mathrm{Pe}(\mathrm{HR}-1)}{\mathrm{Pe}(\mathrm{HR}-1)+1}$$

where **Pe** is the proportion of the source population exposed to a particular risk factor.[8] We used the formula of Greenland to estimate the 95% CIs of adjusted PAFs.[9]

Impact of tobacco smoking on subsequent cancer risk (Figure 1)[10]

Although the relations between tobacco smoking and cancers at various sites are unequivocal, few studies have examined the subsequent risk and PAF of overall cancer incidence in relation to tobacco smoking. This study aimed to develop a relevant epidemiological index of the impact of tobacco smoking on the subsequent risk of cancer in Japan. We conducted a cohort analysis of the possible association between tobacco smoking habits and overall cancer risk among 92 792 subjects (44 521 men and 48 271 women), with a follow-up period of 9.6 years. From 1990 through 2001, there were 4922 incident cases of cancer (2969 men and 1953 women). Responses to the baseline questionnaire indicated that 52.2% of men and 5.6% of women were current smokers, among whom the HR for subsequent cancer occurrence, as compared with never smokers, was 1.64 (95% CI, 1.48–1.82) and 1.46 (1.21–1.75), respectively. The corresponding PAF of overall cancer incidence in men was 22.4% (15.7%–28.5%) and 7.0% (3.7%–10.3%) in relation to current and past exposures to tobacco smoke. In women, the respective PAFs were only 2.2% and 0.6%, due to the low prevalence of current and former smokers. Our results suggest that avoidance of tobacco smoking would prevent 29% of cancers in men and 3% of cancers in women.

Impact of alcohol drinking on overall cancer risk (Figure 2)[11]

In Japan, both alcohol consumption and the proportion of heavy drinkers have been increasing for decades, and alcohol drinking has been recognized as an important and preventable public health problem. The epidemiological background, types of beverages regularly consumed, and genetic polymorphisms for alcohol-related enzymes in Japanese differ from those in Western populations. We conducted a cohort study of alcohol consumption and overall cancer incidence in 73 281 subjects (35 007 men and 38 274 women) aged 40 to 59 years at baseline over a 9.8-year follow-up period. During the period from 1990 through 2001, we identified a total of 3403 cases of newly diagnosed cancer and 1208 cancer deaths. In men, occasional drinkers had the lowest risk of developing cancer, and a positive linear association with ethanol intake was noted: the HRs

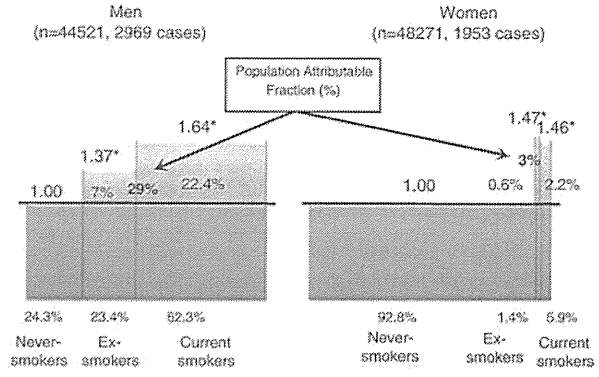

Figure 1. Impact of tobacco smoking on subsequent cancer risk in men and women[10]

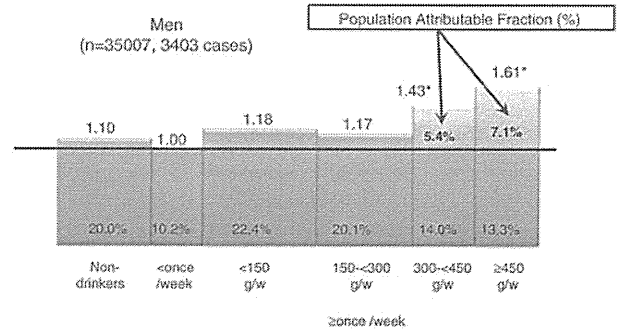

Figure 2. Impact of alcohol drinking on overall cancer risk in men[11]

were 1.18 (95% CI, 0.96–1.44) for 1 g to <150 g per week, 1.17 (0.96–1.44) for 150 g to <300 g per week, 1.43 (1.17–1.75) for 300 g to <450 g per week, and 1.61 (1.32–1.97) for 450 g or more per week (P for trend, <0.001). The positive association was more striking among current smokers and for alcohol-related cancers. Relatively few women were regular drinkers. Our results suggest that ethanol intake elevates the risk of cancer in a dose-dependent manner, and that nearly 13% of cancers among men in this study were due to heavy drinking (≥300 g per week of ethanol), to which smoking substantially contributed. Reduction of smoking is therefore important in decreasing the effect of alcohol on cancer risk.

Impact of BMI on overall cancer risk (Figure 3)[12]

To determine whether BMI extremes in otherwise healthy individuals affect the likelihood that cancer will occur, we conducted a cohort analysis of the possible association between BMI and the risk of overall cancer incidence among 88 927 subjects (42 093 men and 46 834 women), with a 9.5-year follow-up. In men, there was a U-shaped association between BMI and cancer occurrence: men with a BMI of 23 to less than 25 had the lowest risk of cancer occurrence (BMI 14–<19: HR = 1.29, 95% CI = 1.08–1.54; BMI 30–<40: 1.22, 0.92–1.61). This tendency did not change substantially after excluding cases diagnosed early during the follow-up period; cancer mortality showed a similar trend, but with higher risk values. When analyzed according to smoking category, a low BMI had a stronger effect on cancer occurrence in current smokers than in never smokers. There was no marked fluctuation in risk in women. A very low BMI seems to have a greater impact on overall cancer risk in populations with a lower average BMI. Therefore, although

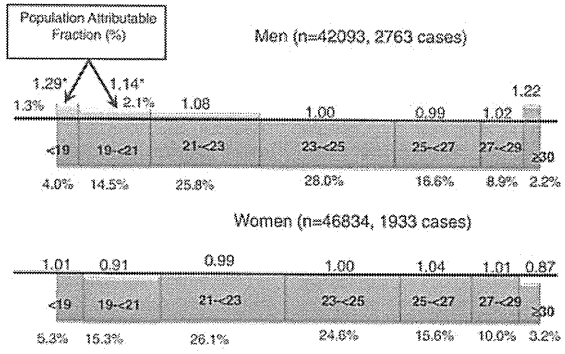

Figure 3. Impact of body mass index (BMI) on overall cancer risk[12]

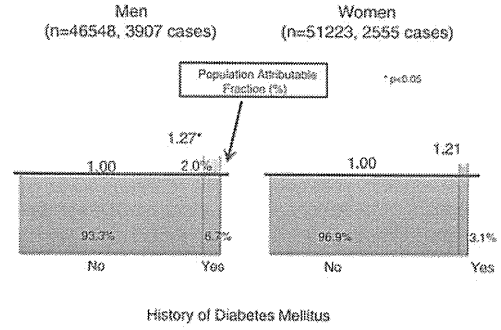

Figure 4. Diabetes mellitus and overall cancer risk[13]

much attention has been paid to the effects of obesity, the health effects at both BMI extremes should be considered in populations with a low average BMI.

DM and the risk of cancer (Figure 4)[13]

As in many other countries, DM is a serious public health problem in Japan. One global estimate projects an increase in prevalence from 6.5% in 1995 to 8.7% in 2025 among Japanese aged 20 years or older. This increase in DM will likely influence trends in related health conditions, including cancer. Clarification of the association between DM and cancer in populations with an increasing DM prevalence, eg, Japanese, is thus a crucial task, not only with respect to causation but also with regard to the formulation of clinical strategies and public health policies for the target population.

We prospectively examined the association between a history of DM and subsequent risk of cancer. A total of 97 771 subjects (46 548 men and 51 223 women) who responded to the baseline questionnaire from 1990 through 1994 were followed up for cancer incidence through 2003. At baseline, 6.7% of men and 3.1% of women had a history of DM. A total of 6462 cases of newly diagnosed cancer were identified. In men, there was a 27% increase in the risk of overall cancer incidence in those with a history of DM (HR, 1.27; 95% CI, 1.14–1.42). The HRs were especially high for cancers of the liver (2.24, 1.64–3.04), pancreas (1.85, 1.07–3.20), and kidney (1.92, 1.06–3.46). We also observed a moderate increase in the risk of colon cancer (1.36, 1.00–1.85) and a borderline significant increase in stomach cancer (1.23, 0.98–1.54). In women, there were borderline significant increases in overall cancer risk (1.21, 0.99–

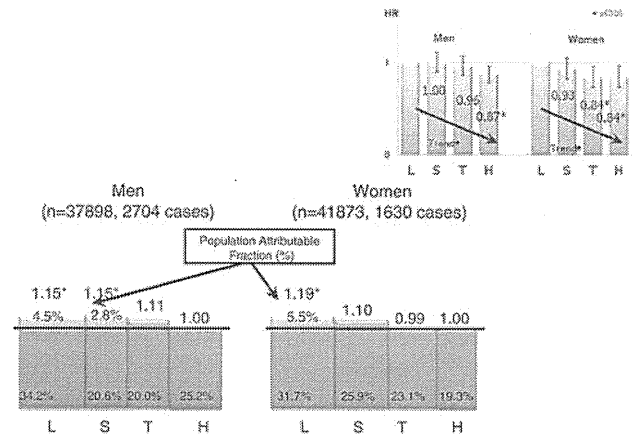

Figure 5. Daily total physical activity level and overall cancer risk[14]

1.47) and ovarian cancer (2.42, 0.96–6.09), and statistically significant increases in the risks for stomach cancer (1.61, 1.02–2.54) and liver cancer (1.94, 1.00–3.73). It appears that, among the general Japanese population, individuals with DM may be at increased risk for overall cancer and for cancer at specific sites.

Daily total physical activity level and overall cancer risk (Figure 5)[14]

A number of investigators have reported that physical activity has beneficial effects on the risk of cancer at specific sites. As a result, physical activity is now regarded as an important target for cancer prevention. At present, however, information on the association between physical activity and overall cancer risk is limited. Given that exercise and physical activity probably affect cancer development at different sites via the same or very similar mechanisms, at least to some degree, it is reasonable to assess the preventive effect of physical activity not only on cancer at specific sites but also on all cancers in aggregate. Further, from a public health perspective, a better understanding of the preventive effect of physical activity on overall cancer risk would provide concrete information for estimating the effects of physical activity measures in health policy planning.

We prospectively examined the association between daily total physical activity (using a score for metabolic equivalents per day) and subsequent cancer risk. A total of 79 771 Japanese men and women aged 45 to 74 years who responded to a questionnaire in 1995 through 1999 were followed for overall cancer incidence (4334 cases) through 2004. As compared with subjects in the lowest quartile, increased daily physical activity was associated with a significantly decreased risk of cancer in both sexes. In men, the HRs for the second, third, and highest quartiles were 1.00 (95% CI, 0.90–1.11), 0.96 (0.86–1.07), and 0.87 (0.78–0.96), respectively (P for trend = 0.005); in women, the HRs were 0.93 (0.82–1.05), 0.84 (0.73–0.96), and 0.84 (0.73–0.97), respectively (P for trend = 0.007). The decrease in risk was clearer in women than in men, especially among the elderly and those who regularly engaged in leisure sports or physical exercise. By site, decreased risks were observed for cancers of the colon, liver, and pancreas in men, and for cancer of the stomach in women. On estimation of the PAF from our results, 4.5% of male cases and 5.5% of female cases were considered preventable if the persons in the lowest physical activity category had increased their activity to a higher level. Increased daily physical activity may thus be beneficial in preventing cancer in a relatively lean population.

Impact of metabolic factors on subsequent cancer risk (Figure 6)[15]

As in many countries, metabolic syndrome has recently attracted substantial attention in Japan, and this is reflected in the government's decision to start a nationwide intervention strategy in April 2008. The National Health and Nutrition Survey in Japan reported that the prevalence of metabolic syndrome in the Japanese population aged 40 to 74 years in 2005 was 25.5% in men and 10.3% in women. Given the expectation that this would likely influence related health conditions, including cancer, clarification of the association between metabolic factors and cancer is a crucial task, not only with respect to causation, but also with regard to the

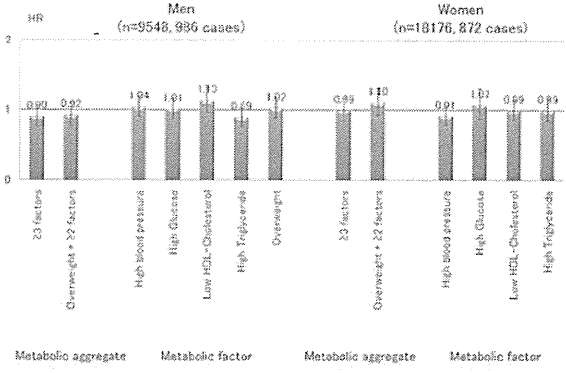

Figure 6. Impact of metabolic factors on subsequent cancer risk[15]

formulation of clinical and public health strategies for the target population. However, the impact of metabolic factors on overall cancer risk has not been clarified.

We prospectively examined whether metabolic factors and their aggregates predict the subsequent occurrence of overall and major sites of cancer. A total of 27 724 participants (9548 men and 18 176 women) aged 40 to 69 years who participated in a questionnaire and health checkup survey in 1993 through 1995 were followed for overall cancer incidence through 2004. HRs and 95% CIs were calculated for metabolic factors (hypertension, high serum glucose, low HDL-cholesterol, hypertriglyceridemia, and overweight) and for 2 aggregates of these criteria (≧3 factors; ≧2 additional factors, plus overweight). In both sexes, the presence of metabolic factors in the aggregate did not predict subsequent occurrence of cancer as a whole. By site, a significant increase in risk was observed for liver cancer in men (≧3 factors: HR, 1.73; 95% CI, 1.03–2.91; and, ≧2 additional factors, plus overweight: 1.99, 1.11–3.58), and pancreatic cancer in women (≧2 additional factors, plus overweight: 1.99, 1.00–3.96). For other sites, positive associations were observed only for specific metabolic factors, namely, hypertriglyceridemia and colon cancer in men (1.71, 1.11–2.62), and obesity and breast cancer in women (1.75, 1.21–2.55). Metabolic factors in the aggregate appear to have little impact on overall cancer risk in the Japanese population, although the association between specific components and specific cancers suggests an etiologic link.

CONCLUSION

We estimated the impact of major risk factors, namely tobacco smoking, alcohol drinking, BMI, history of diabetes, physical activity, and metabolic factors and their aggregates, on overall cancer risk among a Japanese population. Tobacco smoking and heavy alcohol drinking were significantly positively associated with overall cancer risk, and total physical activity was significantly inversely associated with overall cancer risk. In addition, although participants with a history of DM appear to be at increased overall risk of cancer, BMI and metabolic factors in the aggregate had little impact on overall cancer risk in this population.

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APPENDIX

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Gastric Cancer Working Group Report

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Epidemiology: Gastric cancer is the second most common cancer in Asia, more than half of the world's gastric cancer cases arise in Eastern Asia, and the majority of Asia's cases still occur in the distal part of the stomach.
Etiology and Prevention: The etiology of gastric cancer consists of genetic susceptibility, *Helicobacter pylori* infection and environmental risk factors. *Helicobacter pylori* eradication treatment, consumption of fresh vegetables and fruits and use of aspirin and non-steroidal anti-inflammatory drugs seem to reduce the risk of gastric cancer.
Endoscopy and Diagnosis: Screening for gastric cancer is cost-effective in countries with high incidence. Risk stratification may increase the cost-effectiveness of screening in populations at moderate risk. Endoscopic resection is curative in a subset of patients with early cancer.
Surgery and Adjuvant Treatment: R0 resection with D2 lymph node dissection has produced the best survival data. Some kind of post-operative adjuvant chemotherapy including S-1 is recommended after D2 surgery.
Chemotherapy for Advanced Gastric Cancer: As chemotherapy for gastric cancer, fluoropyrimidines plus platinum are the most widely accepted first-line regimens, whereas taxanes or irinotecan are mostly used in second- and third-line settings. Differences in the approval and medical insurance systems may influence the status of these regimens. Trastuzumab in combination with fluoropyrimidines/platinum will be a standard regimen for HER2-positive gastric cancer. Many new targeting agents are currently under investigation, and Asian countries are playing important roles in investigation and development of new and better treatments for this malignancy.

Key words: gastric cancer – Helicobacter pylori – D2 lymphadenectomy – adjuvant chemotherapy – endoscopic treatment – chemotherapy

The Gastric Cancer Working Group report was divided into five chapters: epidemiology, etiology and prevention, endoscopy and diagnosis, surgery and adjuvant treatment and chemotherapy for advanced gastric cancer.

EPIDEMIOLOGY

In spite of the remarkable spontaneous decline in the incidence of stomach cancer in most Western countries, in Asia it is still one of the two most common cancers, following only lung cancer and accounting for 13% of all cancers in Asia (Fig. 1) (1). Estimation of the distribution of gastric cancer in the world in 2002 showed that 56%, more than half of all new cases in the world, occurred in Eastern Asia, with 41% from China and 11% from Japan (Fig. 2) (1). The highest incidences occurred in Korea and Japan. Gastric cancer is relatively common in Asia, Eastern Asia, other Asia, South America and Central and Eastern Europe, whereas it is rare in other European areas and Northern America (Fig. 3) (1). In the common areas, including Eastern Asia, cancer of the distal part of the organ is still the most frequent, whereas the proximal gastric cancer is more common in Western countries (Fig. 4) (2).

In conclusion, gastric cancer is the second most common cancer in Asia, more than half of the world's gastric cancer cases still arise in Eastern Asia, and the majority of those cases still occur in the distal part of the stomach. An increased trend for EC-junction adenocarcinoma is suggested in Western countries, but there is no evidence of such a trend in Asia.

ETIOLOGY AND PREVENTION

Three major factors are involved in the development of gastric cancer: *Helicobacter pylori* infection, genetic

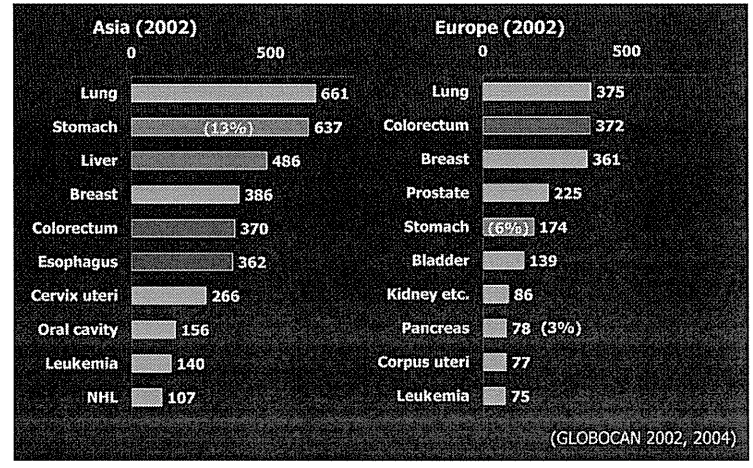

Figure 1. Number of new cases for 10 common cancers (both sexes).

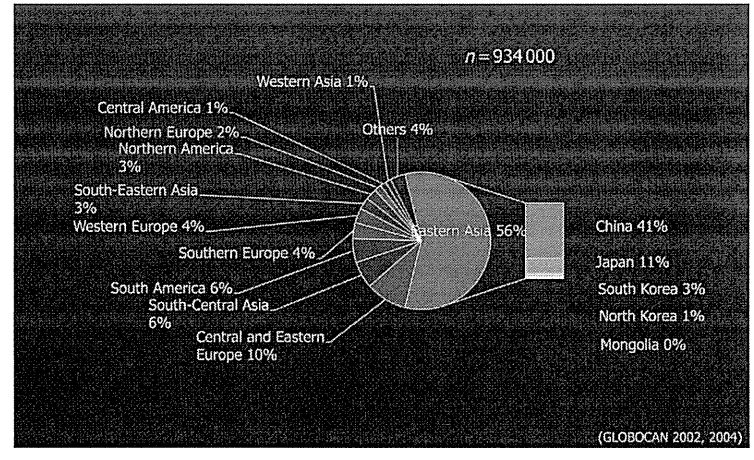

Figure 2. Estimated distribution of gastric cancer in the world in 2002.

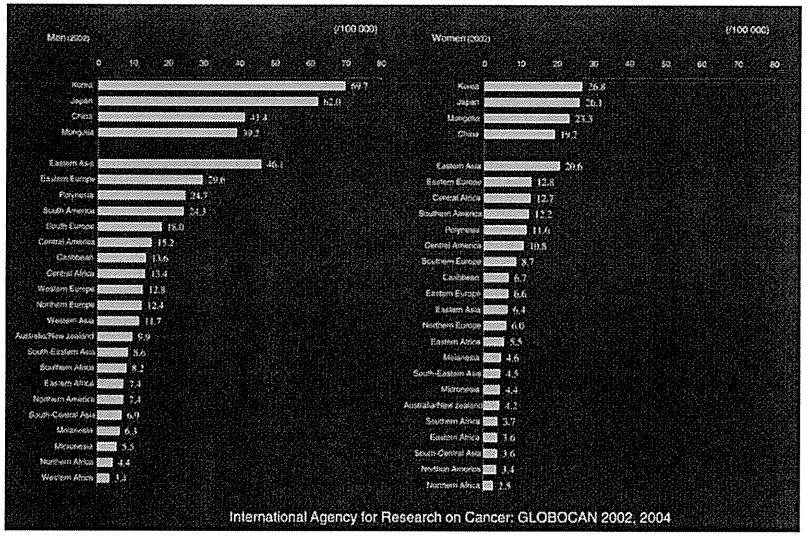

Figure 3. Age-standardized incidence rate of gastric cancer in various area of the world (2002 estimate).

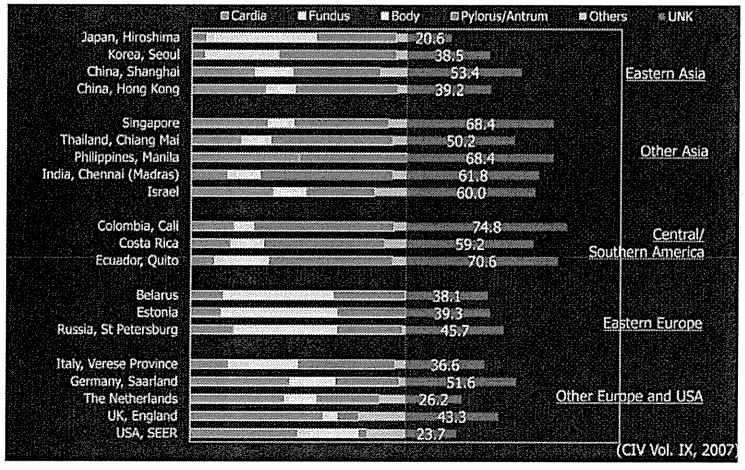

Figure 4. Subsite distribution of gastric cancer, 2000.

susceptibility (CDH1 etc.) and environmental factors (such as smoking, a high-salt diet and low vegetable consumption) (3). *Helicobacter pylori* infection is the most important. A study by Dr Uemura et al. (4), published in the *New England Journal of Medicine*, found no development of gastric cancer in cases without *H. pylori* infection, whereas

2.9% of 1246 cases with *H. pylori* infection developed gastric cancer over a period of 7.8 years. A randomized controlled study in China also showed that *H. pylori* eradication was more effective in patients without atrophic gastritis than those with it (5). Dr Fukase in Japan reported that in a randomized controlled study comparing eradication of *H. pylori* with no eradication after endoscopic mucosal resection (EMR) of early gastric cancer at the 3-year follow-up point significantly reduced the number (9 versus 24) of metachronous gastric cancer developed in the eradication group compared with the control group. It was concluded that prophylactic eradication of *H. pylori* after EMR for early gastric cancer should be performed to prevent the development of metachronous gastric cancers (Fig. 5) (6). These results suggested that it was never too late to eradicate *H. pylori* for prevention of gastric cancer. An Italian group performed a meta-analysis of the published data regarding whether *H. pylori* eradication treatment can reduce the risk of gastric cancer. It was concluded that 1.1%' of treated patients would develop gastric cancer, in contrast to 1.7% of untreated patients. In six studies with about 6700 participants followed for 4–10 years, the relative risk was 0.65, and it was concluded that *H. pylori* eradication treatment seemed to reduce gastric cancer (7). In Taiwan, a nationwide cohort study followed 80 000 patients with *H. pylori*-infected peptic ulcers for 10 years. These patients were divided into early- and late-eradication cohorts. It was concluded that early *H. pylori* eradication showed no significant difference in the gastric cancer risk compared with the general population, but late eradication was associated with an increased risk of gastric cancer. Older age, male gender, gastric ulcer, no regular NSAIDs use and late *H. pylori* eradication represented independent risk factors for gastric cancer development (Fig. 6) (8).

Fock et al. concluded that fruits and vegetables are associated with a reduced risk of gastric cancer in his paper in the *Journal of Gastroenterology and Hepatology*. Supplementation of vitamins and minerals may be unnecessary, at least in healthy subjects with no nutritional deficiencies (9). In a

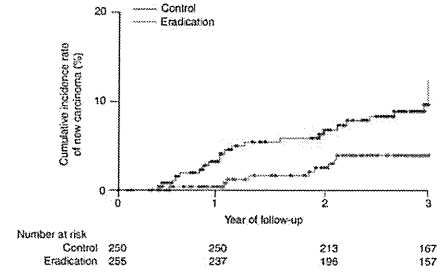

Figure 5. Kaplan–Meier analysis of the cumulative incidence rate of new carcinoma. Source: Fukase et al. (6).

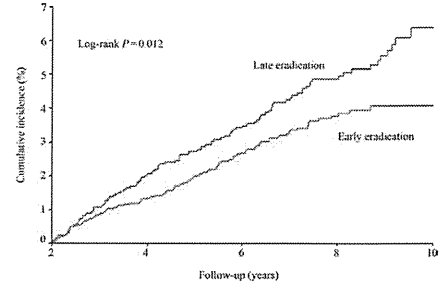

Figure 6. Cumulative incidence of gastric cancer in two groups, early eradication and late eradication groups. Source: Wu et al. (8).

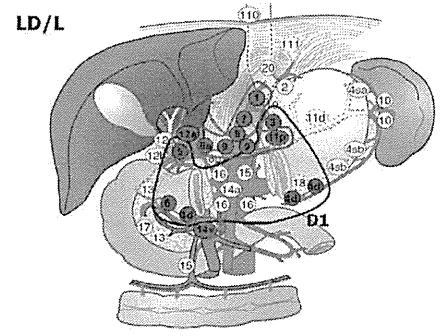

Figure 7. Regional lymph node group according to the location of tumor. Source: Sasako et al. (21) and Yoon and Yang (22).

meta-analysis study, all studies proved that both aspirin and NSAIDs are useful for preventing cardia and non-cardia gastric cancer (10). There is insufficient evidence for any benefit from green tea, vitamins and antioxidants. The biological behaviors of distal and proximal gastric cancers are quite different, but the prevention regimens have been the same, centered on eradication of *H. pylori* infection.

The Working Group concluded that the etiology of gastric cancer consists of genetic susceptibility, *H. pylori* infection and environmental risk factors. *Helicobacter pylori* eradication treatment, consumption of fresh vegetables and fruits and use of aspirin and NSAIDs (11) seem to reduce the risk of gastric cancer.

ENDOSCOPY AND DIAGNOSIS

Experience in Japan has shown that access to screening and early endoscopy increased the proportion of early-stage

gastric cancers, leading to improved survival (12). Cost is a major barrier to screening. Screening is considered to be cost-effective in high-incidence countries, but perhaps not where the incidence of gastric cancer is moderate or low. Risk stratification may help to focus limited resources on patients at greatest risk, and thereby increase the cost-effectiveness of screening (13). Serum pepsinogen-based tests may help to identify a subset of patients with atrophic gastritis, who are especially at a high risk. In a country with high incidence of gastric cancer, such as Japan, it is still very cost-effective to screen even if the cost of endoscopy is high. Singapore and some other countries in East Asia have a moderate incidence of gastric cancer, and screening these populations could be cost-effective if the cost were moderate (13). In Japan, the government-supported screening program has been based on barium, and although very successful, it accounts for less than 10% of all cancers that are diagnosed by screening. Most are detected due to early or easy access to endoscopy, either through outpatient clinics or through health screening outside of the government's screening program (14).

High-quality endoscopy is important and may be facilitated by endoscope preparation, such as lens cleaning, and by patient preparation ahead of endoscopy by the use of defoaming agents, mucolytics and antispasmodics, which make the field of interest much clearer. Techniques such as adequate air insufflation, systematic examination of the entire stomach, use of contrast agents, image enhancement and cognitive training may also help improve yield rates.

Accurate specimen collection and recording of endoscopic findings are important. There is some discordance between Western- and Japanese-trained pathologists in the biopsy definition of early gastric cancer. In the West, the gold standard for diagnosing cancer is to detect invasion of tumor cells into the lamina propria, muscularis mucosae or submucosal layer, whereas in Japan, it is more important to detect cellular atypia or structural atypia, regardless of invasion, when making a diagnosis of cancer. The revised Vienna classification has helped resolve some of these differences and may be a good starting point for consensus between Western and Japanese pathologists (15).

Gotoda et al. (16) reported that there is a clearly defined subgroup of patients with early gastric cancer that has a virtually negligible risk of nodal metastasis. Such patients could be treated definitively by local resection, with the expected long-term outcome equivalent to radical surgery. Further development led to the expanded criteria for endoscopic therapy of early gastric cancer, with *en bloc* resection being the primary goal (17). Endoscopic resection can be considered curative if the lesion shows differentiated histopathology, is limited to the mucosal layer or <500 μm submucosal invasion, with clear vertical and lateral margins, and no lymphovascular involvement. EMR has the advantages of short procedure time and low risk of perforation, which make it an attractive option for small lesions. EMR for differentiated, non-ulcerated early cancer <20 mm in diameter is associated with an excellent 10-year survival rate of 99% (18). Endoscopic submucosal dissection (ESD) is associated with a lower local recurrence rate than EMR because the technique permits *en bloc* resection without size limitation. Procedure times for ESD are longer, however, with higher delayed bleeding and perforation risk (19). A recent long-term follow-up study showed that ESD for early gastric cancer, which met the expanded criteria, resulted in 5-year overall and disease-specific survival rates of 97% and 100%, respectively (20). Training opportunities in ESD for endoscopists from outside Japan and Korea, however, remain limited.

In conclusion, screening for gastric cancer is cost-effective in countries with high incidence. Risk stratification may increase the cost-effectiveness of screening in populations at moderate risk. Barium meal-based screening is government-funded in Japan, but is less accurate than gastroscopy. Gastroscopic screening is desirable in high-risk populations. High-quality endoscopy may increase diagnostic yield in early cancer. Endoscopic resection is curative in a subset of patients with early cancer as defined by the expanded criteria. EMR has shown long-term outcomes comparable with surgery in patients with small lesions, and similar outcomes with ESD for larger lesions in experienced hands. Standardization between Western- and Japanese-trained pathologists in diagnosing gastric cancer is urgently needed. Structured training programs for ESD should be set up in high-volume centers and made accessible to suitable regional candidates.

SURGERY AND ADJUVANT TREATMENT

For gastric cancer, so-called D1, or perigastric lymph node, dissection is common in Western countries, whereas in high-incidence countries like Japan and Korea, so-called D2 dissection is considered to be the standard (Fig. 7) (21,22).

An RCT from UK comparing D1 versus D2 found very high mortality but failed to show a difference (23,24). The trial was flawed due to the very high mortality, inclusion of a large proportion of stage I and absence of any description regarding the quality of lymph node dissection. A Dutch trial started 20 years ago also showed much higher mortality for D2 compared with D1 dissection and demonstrated no survival benefit (25,26). These two trials were closed before reaching the plateau of the learning curve, and the high post-operative mortality offset the effect of the D2. D2 dissections should be carried out in specialized centers.

An RCT in Taiwan compared D1 and D2 showed survival benefit of D2 dissection with reasonable morbidity and mortality (Fig. 8) (27).

To investigate even more extensive dissection of gastric cancer, a Japanese group compared D2 with D2 plus para-aortic nodal dissection (28,29). The results showed slightly higher morbidity, but without increase in mortality. These morbidity and mortality results were acceptable. However,

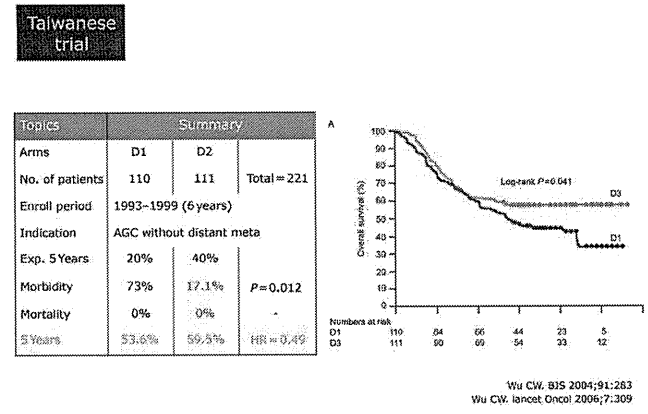

Figure 8. Nodal dissection for patients with gastric cancer: a randomized controlled trial. Source: Wu et al. (27).

no survival difference was observed, and D2 was thus the optimal surgery in that RCT. Comparison of reports from various countries reveals that the mortality is higher when the volume is lower, again demonstrating that D2 dissection should be performed in high-volume and/or specialized centers.

Regarding the role of adjuvant treatment, a major trial in Europe showed survival benefit from perioperative chemotherapy, but less than half of the patients underwent D2 dissection and the study also included esophageal cancer cases (30).

An RCT performed in the USA investigated the role of post-operative chemoradiotherapy and also showed significant survival benefit (31,32). However, only 10% of the patients underwent D2 dissection, there was a very high rate of local recurrence, and the surgery was not standardized among the participating hospitals. Subgroup analysis found survival benefit only in D0 or D1, but not in the D2-dissected group. The study thus showed that D0/D1 dissection was insufficient treatment.

In a Japanese randomized trial, curative D2 dissection alone was compared with D2 followed by post-operative chemotherapy by oral S-1 (33). In contrast to the Western studies, almost all of the cases in this study underwent D2 dissection, and the 3-year survival rate showed a 10% improvement (Fig. 9). A clinical trial of adjuvant treatment is being conducted in Korea, China and Taiwan, and 1024 cases have been enrolled. The results will be available within a few years. A Japanese group and a Korean group are working together to assess, for the first time, the role of reductive gastrectomy in Stage IV gastric cancer treatment (34). The chemotherapy applied in both arms is S-1 plus cisplatin. Although a very difficult project, it is very important, and it is hoped that other Asian countries will join this collaboration in the future.

In conclusion, with regard to the extent of surgery, R0 resection with D2 lymph node dissection has produced the best survival data. Some kind of post-operative adjuvant chemotherapy including S-1 is recommended after D2 surgery. In areas with a high incidence of gastric cancer, the quality of treatment can be kept very high, with both endoscopic treatment and surgery. At the moment, at least in Asia, D2 dissection should be considered as the standard.

CHEMOTHERAPY FOR ADVANCED GASTRIC CANCER

There are now four active cytotoxic agents for advanced gastric cancer, consisting of fluorouracils, platinum, taxanes and irinotecan. The fluorouracils include 5-FU, S-1 and capecitabine, and the platinum include cisplatin and oxaliplatin. During the last decade, various randomized trials investigated the optimal combination of these four chemotherapy drug groups in Japan, Korea and China (Table 1). Capecitabine plus platinum was at least non-inferior to 5-FU plus cisplatin in terms of survival (35,36). S-1 plus cisplatin showed a comparable median time to progression to those in capecitabine or 5-FU plus cisplatin in Western studies (37,38), whereas the Japanese studies yielded relatively longer survival than the Western studies. These favorable survival in Japanese studies compared with the Westerns might be caused by longer survival after failure of the first-line therapy associated with higher rates of subsequent therapy than in the Western studies (Fig. 10).

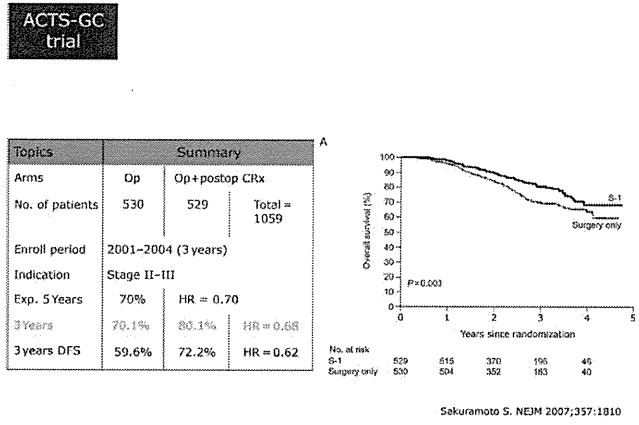

Figure 9. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. Source: Sakuramoto et al. (33).

Table 1. Results of randomized trials using newer regimens: advanced gastric cancer

Study	Treatment	*n*	RR (%)	MTTP (months)	MST (months)	*P* value[a]
V325 (JCO2006)	CDDP + FU (CF)	230	25	3.7	8.6	
	Docetaxel + CDDP + FU (DCF)	227	37	5.6	9.2	0.02
V306 (ASCO2005)	CDDP + FU (CF)	163	26	4.2	8.7	
	CPT-11 + FU (IF)	170	32	5.0	9.0	NS
ML07132 (ASCO2006)	FU + CDDP (FP)	156	29	5.0	9.3	
	Capecitabine + CDDP (XP)	160	41	5.6	10.5	NS
JCOG9912 (ASCO2007)	FU	234	9	2.9[b]	10.8	
	S-1	234	28	4.2[b]	11.4	NS
	CPT-11 + CDDP	236	38	4.8[b]	12.3	NS
SPIRITS (ASCO2007)	S-1	150	31	4.0[b]	11.0	
	S-1 + CDDP	148	54	6.0[b]	13.0	0.037
TOP002 (ASCO-GI2008)	S-1	162	27		10.5	
	S-1 + CPT-11	164	42		12.8	NS

[a]Test for superiority in OS.
[b]PFS.

The approval status of active agents for gastric cancer differs among four East Asian countries. Capecitabine and oxaliplatin are not yet available in Japan, and S-1 and oxaliplatin are not available in Taiwan (Table 2). In Japan, approval is always associated with medical reimbursement, but that is not always the case in other countries. The differences caused by the medical insurance systems may affect the survival results larger than by ethnic differences in biology or pharmacokinetics. In countries with limitations on medical reimbursement for second- or further line chemotherapy, such as Western countries and Asian countries other than Japan, triplet regimen such as docetaxel + cisplatin + 5-FU is becoming more popular. However, in Japan, all agents that have been approved are covered by medical reimbursement at any line of chemotherapy, which cause that FUs plus platinum are the most popular first-line

Table 2. Approval status of active agents in gastric cancer

Agents	Japan	Korea	China	Taiwan
5-FU	○	○	○	○
S-1	○	○	○	×
Capecitabine	×	○	○	○
Cisplatin	○	○	○	○
Oxaliplatin	×	○	○	×
Paclitaxel	○	○	○	○
Docetaxel	○	○	○	○
Irinotecan	○	○	○	○

○, medical reimbursement in Japan; ×, medical reimbursement in ex-Japan.

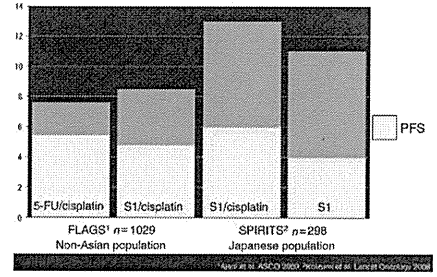

Figure 10. Survival in advanced gastric cancer: Japanese versus Western population.

regimens followed by taxane or irinotecan. In conclusion, no global standard regimen has been established yet as the first-line standard chemotherapy for metastatic cancer. In Asian countries, FU and platinum combinations are the most widely used regimens, with median progression-free survivals of 5–6 months. Differences in the approval and medical insurance systems may influence the status of these regimens.

The ToGA study compared the cytotoxic combination (5-FU or capecitabine + cisplatin) with and without trastuzumab in patients with HER2-positive gastric cancer (Fig. 11) (39). This is a global randomized trial, but more than half of the patients have been recruited from East Asian countries, including Korea, Japan and China. Trastuzumab showed a significant survival advantage compared with the cytotoxic agent combinations, with a hazard ratio of 0.74. From the Asian point of view, the ToGA trial indicates that trastuzumab in combination with FU/platinum will be a new option for HER2-positive gastric cancer. Moreover, the HER2-positive population will become an independent entity, as in breast cancer, although further studies are needed. Regional

Table 3. International investigational new drug registration randomized controlled trials for metachronous gastric cancer: leading countries

Agents	Study name	Leading country	Region	Enrollment status
Trastuzumab	ToGA	Korea	Asia, EU, SA	Published
Bevacizumab	AVAGAST	Japan	Asia, EU, N/S A	Completed
Cetuximab	EXPAND	Germany	EU, Asia	Recruiting
Lapatinib (first line)	LOGiC	Korea	Asia, EU, N/S A	Recruiting
Lapatinib (second line)	TYTAN	Japan	Asia	Recruiting
Panitumumab	REAL3	UK	EU	Recruiting
Everolimus	GRANITE-1	Japan	Asia, EU, N/S A	Recruiting

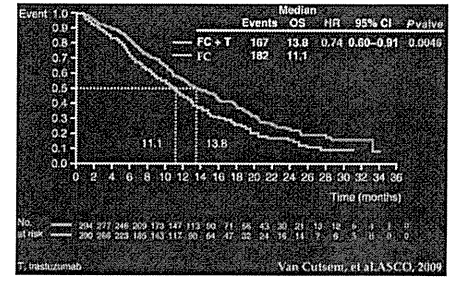

Figure 11. Overall survival results in ToGA trial.

differences, such as the HER2-positive rate, may be clarified by further analyses. Five of seven ongoing global RCTs for metastatic gastric cancer are led mainly by Japan and Korea. Asian countries are playing a major role in the development of new agents for gastric cancer (Table 3) (40).

In conclusion, FUs plus platinum are the most widely accepted first-line regimens for gastric cancer, whereas taxanes or irinotecan are mostly used in second- and third-line settings. Differences in the approval and medical insurance systems may influence the status of these regimens, and the improvement in these status is hopefully done in many countries. Trastuzumab in combination with FUs/platinum will be a standard regimen for HER2-positive gastric cancer, and the recent phase II/III trials showed favorable median survival times exceeding 1 year. Many new targeting agents are currently under investigation and the roles of Asian countries in the development of new agents will become important.

Conflict of interest statement

None declared.

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Cancer Registry and Epidemiological Study Working Group Report

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International Agency for Research on Cancer: The International Agency for Research on Cancer serves as a global reference for cancer information. The Cancer Information Section of the International Agency for Research on Cancer publishes the world's largest information database on cancer incidence and supports cancer registries by providing administrative facilities and training, etc. Many Asian countries have published cancer registries, but Indonesia and Bangladesh have yet to do so.
International Association of Cancer Registries: The International Association of Cancer Registries is a non-governmental organization that promotes information exchange between cancer registries internationally. It supports cancer registries by means of fellowship funds and computer programs.
Cooperative Studies: Asian cooperative studies using cancer registration data are essential for combating cancer in the region. For a cooperative study, countries first need to exchange cancer data and then conduct a comparative study using non-individualized data. The third step is collection of individualized, anonymous data, which would improve comparability.
Collaborative Epidemiological Studies: The Asia Cohort Consortium, which includes investigators from various countries, is a complicated collaboration. Good epidemiological research collaboration requires researchers' comprehension of the significance of multinational collaborative studies, good coordination, adequate funding and balanced collaboration.
Conclusions: Asia faces various problems in relation to cancer registry, including inadequate quality, weak infrastructure, insufficient coverage, etc. Epidemiological studies are hampered by differences in expertise and resources, limited understanding of epidemiology, etc. To alleviate those problems, an organization for Asian cooperation on cancer registration should be established. Adequate funding of registries and activities is essential. Collaborative and comparative epidemiological studies based on data from cancer registries are needed.

Key words: cancer registries – epidemiological studies – collaboration – network

The Cancer Registry and Epidemiological Study Working Group comprised almost 50 members from 19 countries. Its discussions focused on the registry systems and collaborative work necessary for attacking the problem of cancer in the Asia-Pacific region.

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

The International Agency for Research on Cancer (IARC)'s mission is cancer research for cancer prevention. It also serves as a global reference for cancer information, including geographical variations, incidence and trends over time. The IARC also provides education and training for low-resource countries. The Cancer Information Section (CIS) includes three groups: biostatistics, data analysis and descriptive epidemiological production. One of the core activities of the CIS is to issue the cancer incidence in five continents series, which is the world's largest database of information on cancer incidence and has been invaluable for conducting cancer research, establishing cancer control programs and determining healthcare policies around the world. The CIS also supports cancer registries by providing administrative facilities, conducting site visits, providing individual and group training, etc. In 2009, Asian Workshops were held in Vietnam and Bhutan.

Various Asian countries have published cancer registries over recent years. Data from 77 registries in 18 countries were submitted for inclusion in the IARC's *Cancer Incidence in Five Continents Vol. IX*, and 44 (55%) of those registries in 15 countries were accepted. (1) Sixty per cent of the world's population lives in Asia, and 6 of the 10 most-populated countries are in Asia, consisting of China, India, Indonesia, Pakistan, Bangladesh and Japan. Unfortunately, there has still been no cancer registry data from two of those Asian countries, Indonesia and Bangladesh (Table 1).

GLOBOCAN 2002 estimated 4.8 million cases of cancer and 3.4 million deaths in Asia, representing almost 45 and 50%, respectively, of the world's cases. (2) GLOBOCAN data are being updated, and the objective is to provide estimates of cancer incidence, mortality and prevalence for 28 major cancers. Estimated data for 2008 showed that the number of cancer cases in Asia had increased by ~10% since 2002, but deaths increased only slightly.

INTERNATIONAL ASSOCIATION OF CANCER REGISTRIES

The International Association of Cancer Registries (IACR) is a non-governmental organization that was founded in 1966 to foster the exchange of information between cancer registries internationally, aimed at improving the quality of data and comparability between registries. The number of member countries has been increasing, especially Asian nations. In 2009, members from 26 countries covered ~20% of the world's population. The IACR is affiliated with two scientific journals, the European Journal of Cancer Prevention and the Asian Pacific Journal of Cancer Prevention. The IACR standards have been presented in a number of publications, aimed at improving the quality of data and comparability between registries. The IACR provides support to cancer registries by means of fellowship funds (the Calum Muir Memorial Fellowship and the Constance Percy Memorial Fund) and also computer programs. Many Asian countries have cancer registries, but some do not, including North Korea, Cambodia and Laos. Meetings to set up an Asian Network of Cancer Registries were held in Korea in 2008 and Thailand in 2009. Then a survey was conducted regarding the establishment of an Asian Network of Cancer Registries, and 22 responses were obtained from 109 Asian registries (Fig. 1). Seven main objectives of networking were favored for the organization, including training for standardization of networking, planning and execution of collaborative research, evaluation of cancer control and treatment outcomes, meetings and discussions, etc. Regarding the name, half of the respondents preferred 'Asian Association of Cancer Registries', whereas the other half preferred 'Asian Network of Cancer Registries'.

Table 1. Cancer Registries in Asia

Eastern (6)	South-Eastern (11)	South-Central (14)	Western (18)
China: 43 + (A)	**Brunei**: N	Afghanistan	Armenia
Japan: 35 + (A)	Cambodia	**Bangladesh**	**Bahrain**: N
South Korea: 8 + N	**Indonesia** (H)	**Bhutan**: N	**Cyprus**: N
North Korea:	Lao	**India**: 10 (A)	**Israel**: N
Mongolia: N	**Malaysia**: 2 + N	**Iran**: 2	**Jordan**: N
Taiwan: N	Myanmar	Kazakhstan	**Kuwait**: N
	Philippines: 4	Kyrgyzstan	**Oman**: N
	Singapore: N	Nepal: 2	**Turkey**: 2
	Timore	**Pakistan**: 2	Others:
	Thailand: 19 + (A)	**Sri Lanka**	
	Vietnam: 6	Others	

Bold: Countries where registries are in operation.

DESIGNING COOPERATIVE STUDIES

A major element in the overall strategy for combating cancer in Asia-Pacific countries in the future is the effective design and execution of cooperative studies using cancer registration data and international comparisons with Asian countries. The rationale is that society, the mass media and health authorities pay more attention to cancer incidence and trend data when they are compared with other countries, rather than only within their own country. Moreover, the results contribute to improved cancer control planning in the participating countries.

Prior to a cooperative study, countries need to exchange data regarding cancer in each of their countries. In Japan, the incidence of hepatocellular carcinoma (HCC) has been decreasing because of reduced hepatitis C virus (HCV) infection rates due to improved hygiene and prevention of

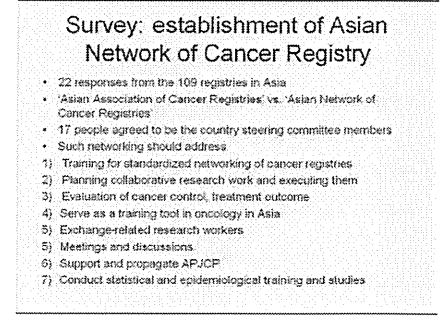

Figure 1. Survey: Establishment of Asian Network of Cancer Registry.