Table 5 Comparison of smoking index of ever-smokers according to the rs5320 polymorphism of *DBH*

	Males			*Females*		
Index	n	*Mean ± s.d.*	P-*value*[a]	n	*Mean ± s.d.*	P-*value*[a]
Age at start of smoking						
GG	1062	19.6 ± 3.3		62	33.9 ± 13.2	
AG	268	19.9 ± 4.3	0.593	19	33.9 ± 10.1	0.949
AA	16	20.4 ± 3.0		1	35.0	
CPD						
GG	1065	21.3 ± 13.1		63	13.8 ± 8.4	
AG	268	20.9 ± 12.5	0.007	19	11.2 ± 6.6	0.389
AA	16	13.4 ± 6.1		1	20.0	
CPD × years						
GG	1061	862 ± 598		62	419 ± 377	
AG	268	840 ± 528	0.055	19	334 ± 289	0.557
AA	16	549 ± 226		1	620	
FTND						
GG	983	3.62 ± 2.23		57	2.60 ± 2.02	
AG	251	3.53 ± 2.07	0.044	19	1.53 ± 1.84	0.030
AA	15	2.20 ± 1.42		1	4.00	
TDS						
GG	934	3.08 ± 2.47		51	2.94 ± 2.48	
AG	229	3.10 ± 2.57	0.215	17	2.59 ± 2.58	0.692
AA	15	1.87 ± 1.46		1	4.00	
Times of trial for quitting smoking in current-smokers						
GG	276	1.37 ± 1.68		17	1.38 ± 1.69	
AG	64	1.34 ± 1.48	0.415	7	0.71 ± 1.11	0.557
AA	3	0.33 ± 0.58		1	2.00	
Times of trial for quitting smoking before succeeding in ex-smokers						
GG	698	2.10 ± 1.54		35	1.74 ± 1.28	
AG	182	2.12 ± 1.52	0.916	12	1.42 ± 1.17	0.317
AA	12	2.17 ± 1.59		0	–[b]	

[a]Kruskal–Wallis test.
[b]Not applicable.

investigated as Zabeitian *et al.*[23] reported this polymorphism as a functional polymorphism explaining inter-individual difference of plasma DBH activity. As to its possible association with smoking behaviour, Freier *et al.*[24] reported that individuals who had at least one *DBH*-1021 T allele smoked fewer cigarettes per day than CC homozygotes in relatively small numbers of the European smokers ($n = 220$). Another polymorphism, *DBH* polymorphism T1368A (rs77576840), has been shown to be associated with cigarette consumption.[25]

The tobacco and genetics consortium did a meta-analysis totalling 74 053 subjects and found *DBH* rs3025343 [G] is associated with smoking cessation.[26] Siedlinski *et al.*[27] reported the genome-wide study of chronic obstructive pulmonary disease. They could replicate the result by the above consortium; that is, there was an association between a candidate genotype rs3025343 and smoking cessation in their subjects. The other polymorphisms of *DBH* have been also attempted to correlate with smoking. For example, the rs77905 was investigated in terms of the association with smoking status and nicotine level in 1518 adolescent subjects in United Kingdom, but no association was found.[28] Actually, Breitling *et al.*[29] reported this rs77905 polymorphism did not influence smoking cessation programme including 577 heavy smokers.

The rs5320 polymorphism of *DBH* was investigated as haplotype analysis of Parkinson's disease. Haplotypes rs1611115T > C–rs1108580A > G–rs5320A > G–rs129882C > T are reported to be associated with Parkinson's disease.[13] Our findings that *DBH* rs5320 genotype are associated with smoking behaviour remind us of a well-known observation that Parkinson's disease is less prevalent in smokers and there may be a common genetic root for these status, Parkinson's disease and (addicted) smoking.[30–32] Haplotype block structure of Japanese and Han Chinese population is shown in the Figure 1. Actually, this haplotype block indicates the relatively strong linkage ($r^2 = 0.63$) between rs1108589 and rs5320. Among the single-nucleotide polymorphisms mentioned above, the rs77576840 is not so common and is not listed in the HapMap database. It is between rs302530 and rs1108580. The rs3025343 is outside the figure covers (far 5′ upstream).

As shown previously, numerous polymorphic sites of *DBH* were investigated in various populations. We picked up rs5320 because the prevalence in Japanese is feasible and this is a non-synonymous variation. These polymorphic sites including rs5320 are linked with each other depending on populations. Most of them do not have mechanistic rationale for why these polymorphisms are apparently associated with smoking behaviour, which awaits further investigation. The polymorphism at rs5320, G allele in GCG (Ala) vs A allele in ACG (Thr) at the position 211 of this protein may not have a severe biological effect probably, considering these two amino acids exist alternatively from each other in some of the primates (Figure 2). In regard to our result that males with AA genotype had the lowest CPD and FTND score, the reason why this A allele behaves in a recessive manner is unknown. Effect of amino-acid substitution may influence only when both alleles are variants. An exploration on functional rationale would be warranted.

Interestingly, unlike the FTND scores, no relation was found between the TDS and any of the rs5320 allelotype in our study. This could be due to the fact that the traits detected by the scores of the FTND and TDS are different from each other.[33] Smoking is a personal behaviour that can be attributed to many genetic and environmental factors. The questions in the FTND detect the physical aspects of nicotine dependence, including the one on the value of CPD, whereas those in the TDS tend to focus on the mental aspects of smoking. The TDS is a questionnaire for screening tobacco/nicotine dependence according to some criteria of mental disorder. These characteristics of scales may have led to the result that the CPD and FTND, not TDS in males, were related to the DBH polymorphism significantly. Regarding female subjects, the number of smoker with AA genotype was only one, so the relations between the FTND and this polymorphism should be examined further.

Limitations of this study and its interpretation include the fact that the participants were recruited from a rural city, where demographical and occupational characteristics are different from those living in urban cities or agricultural communities. To validate our observations, replication of this study would need to be done with larger and different populations. This would allow for a larger representation of the various genotypes associated with the *DBH* rs5320 polymorphism and also a larger female sample size, so that one can be able to make a clearer conclusion whether the AA genotype of this locus corresponds

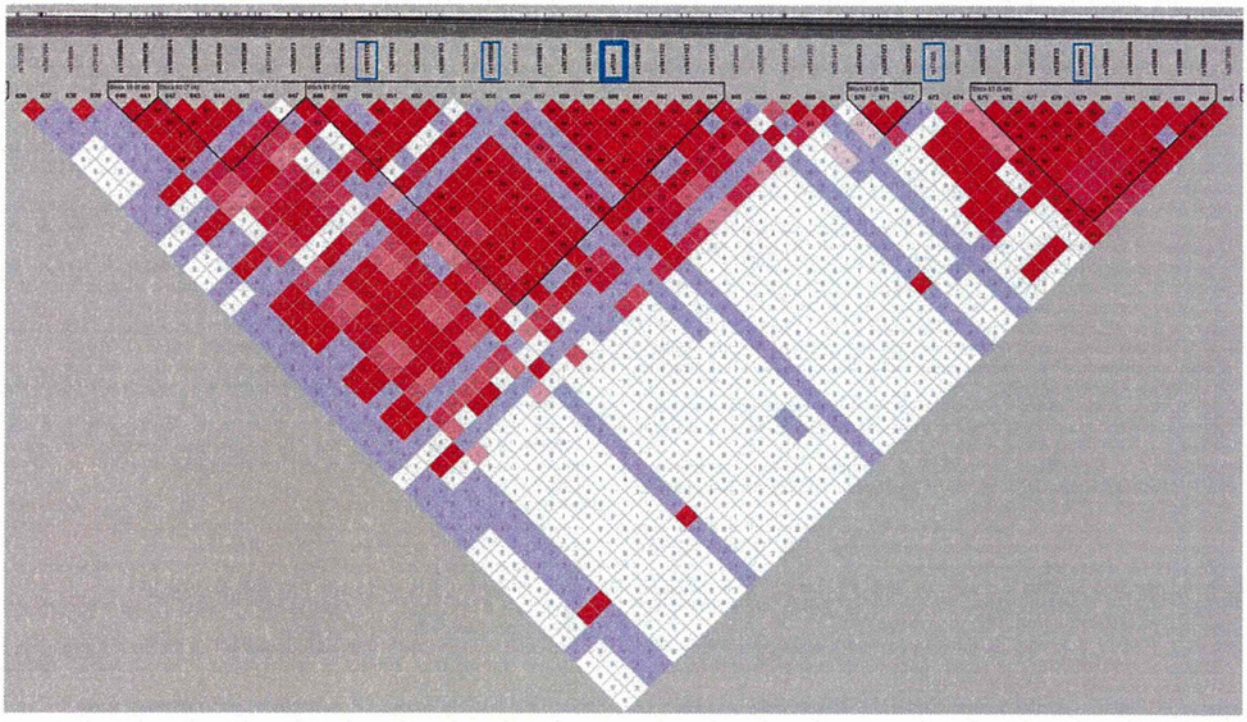

Figure 1 The state of linkage disequilibrium (LD) between the single-nucleotide polymorphisms in the region around the rs5320 polymorphism, which is shown in thick rectangle. The rs1611115, rs77905, rs129882 and rs1108580 are shown in thin rectangles. The strength of LD was calculated based on the genotype data of the Japanese and Han Chinese population extracted from the position 135,474,113 to 135,522,004 on chromosome 9 in the Hapmap database (http://hapmap.ncbi.nlm.nih.gov/index.html.ja).[34] Numbers in squares represent percentage of the r^2 values. Squares without numbers represent $r^2 = 1$. The colour scheme was according to the 'Standard Color Scheme' of the Haploview v.4.1 software (Lod $\geqslant 2$, $D' = 1$: bright red; Lod $\geqslant 2$, $D' < 1$: shades of pink/red; Lod < 2, $D' = 1$: blue; Lod < 2, $D' < 1$: white).[35] The LD blocks were defined based on the default algorithm by Gabriel *et al.*[36]

Homo sapiens	P	S	D	A	C	T	M
Pan troglodytes	P	S	D	A	C	T	M
Pongo abelii	P	S	D	T	Y	T	M
Nomascus leucogenys	P	S	D	A	C	T	M
Macaca mulatta	P	S	D	T	Y	T	M

rs5320, exon4

Figure 2 Amino-acid alignment surrounding the position 211 in human and the corresponding positions in the other primates. Though the information on genetic polymorphism in the primates is limited, the database shows this position is Ala or Thr in several primates including human being.

with having a higher FTND score. Recruitment of sample population from a wider demography that is, urban cities and agricultural villages would be needed. This would take into account the different smoking characteristics. Despite these shortcomings, our data has provided a major clue in understanding the smoking behaviour of humans.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Lipid peroxidation-induced DNA adducts in human gastric mucosa

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DNA adducts are a major cause of DNA mutation and DNA mutation-related diseases, but the simultaneous identification of multiple DNA adducts has been a challenge for a decade. An adductome approach using consecutive liquid chromatography and double mass spectrometry after micrococcal nuclease treatment has paved the way to demonstrations of numerous DNA adducts in a single experiment and is expected to contribute to the comprehensive understanding of overall environmental and endogenous exposures to possible mutagens in individuals. In this report, we applied an adductome approach to gastric mucosa samples taken at the time of a gastrectomy for gastric cancer in Lujiang, China, and in Hamamatsu, Japan. Seven lipid peroxidation-related DNA adducts [1,*N6*-etheno-2′-deoxyadenosine, butanone-etheno-2′-deoxycytidine (BεdC), butanone-etheno-2′-deoxy-5-methylcytidine, butanone-etheno-2′-deoxyadenosine (BεdA), heptanone-etheno-2′-deoxycytidine, heptanone-etheno-2′-deoxyadenosine (HεdA) and heptanone-etheno- 2′-deoxyguanosine] were identified in a total of 22 gastric mucosa samples. The levels of these adducts ranged from 0 to 30 000 per 10^9 bases. Although the presence of *Helicobacter pylori* DNA in the mucosa was not related to these adducts level, the levels of BεdC, BεdA and HεdA were higher in the Japanese gastric mucosa samples. The profiles of these 7 adduct levels among the 21 cases were capable of discriminating between the possible origins (China or Japan) of the gastric mucosa samples. Our report is the first demonstration of lipid peroxidation-related DNA adducts in the human stomach, and these observations warrant further investigation in the context of the significance of DNA adducts in human gastric carcinogenesis.

Introduction

DNA adducts are a major cause of mutation (1); thus, the recognition of adducts in target organs in individuals provides very basic information on the susceptibility to and the initiation of cancer in particular organs and particular individuals (2).

Abbreviations: BεdA, butanone-etheno-2′-deoxyadenosine; BεdC, butanone-etheno-2′-deoxycytidine; BεmedC, butanone-etheno-2′-deoxy-5-methylcytidine; εdA, 1,N6-etheno-2′-deoxyadenosine; HεdA, heptanone-etheno-2′-deoxyadenosine; HεdC, heptanone-etheno-2′-deoxycytidine.

The identification of DNA adducts has been a technical challenge for several decades (3), but recent innovations have enabled us to identify multiple DNA adducts in human tissues (4,5). This adductome approach has successfully revealed a considerable number of lipid peroxidation-related adducts in human tissues, including human lungs (6). The human stomach is another organ that is almost directly exposed to the environment and especially to dietary substances with the potential to damage the DNA of the host on a daily basis. Because dietary carcinogens and mutagens have been identified in ordinary food and/or food additives (7), DNA adducts related to such environmental carcinogens are probably to be present in human gastrointestinal tracts, especially in patients who suffer from cancers of the gastrointestinal tract. In addition, the gastric mucosa is a site where chronic inflammation may occur, especially in individuals infected with *Helicobacter pylori (H. pylori)* (8–10); thus, inflammation-related DNA adducts may exist in the gastric mucosa, possibly playing an important role in gastric carcinogenesis (11). Previously, several attempts have been made to detect a variety of DNA adducts in the stomach of experimental animals (12,13) and human beings (14). Among them, Abdul-Momen and colleagues detected five DNA adduct spots using the P^{32}-postlabeling method (15). They claimed that these five DNA adducts were specific to the gastric mucosa in gastric cancer patients and were absent in newborn stomach. This observation, however, has not been followed-up to date, probably because the P^{32}-postlabeling method cannot further delineate the molecular species in DNA from the gastric mucosa. Recently, our group established a method consisting of liquid chromatography followed by double tandem mass spectrometry to identify numerous adducts in human and animal tissues (4–6,16) during a single experiment. In contrast with the P^{32}-postlabeling method, this method directly facilitates the identification of modified DNA bases by placing the standard substances in parallel. Several adducts with different chemical formulae and mass-per-charge values have been confirmed to exist in human tissues (4,6). Recently, Marsden and colleagues (17) used liquid chromatography and accelerator mass spectrometry and showed a dose-response relationship with the quantity of a specific DNA adduct (N7-(2-hydroxyethyl)guanine, a presumed cancer-causing adduct) in an experimental model of stomach cancer; they stated that their method and calibration would be useful for the study of human tissues and epidemiology frameworks. However, they have not yet reported any information specific to human gastrointestinal tissue.

In this report, we applied the adductome approach to human stomach samples obtained during gastrectomies for the resection of gastric cancer and validly identified seven different lipid peroxidation-derived DNA adducts in the human stomach for the first time.

Materials and methods

DNA extraction from gastric mucosa

Gastric mucosa was taken from non-tumor tissue sections of stomachs resected for the treatment of gastric cancer at Lujiang Hospital, China, and at Hamamatsu University Hospital, Japan. For sample quality control, pathologists from each institution made a site-visit and confirmed that non-tumor, mucosal portions were immediately obtained after resection and were frozen in liquid nitrogen and kept at −70°C in a freezer until DNA extraction. The clinicopathological profiles of the cases are shown in Table I. For all the samples, DNA extraction was conducted according to the same protocol and by the same person (N.K.) without knowledge of the origin (Lujiang or Hamamatsu) of the tissue using a Gentra Puregene™ Tissue Kit (Qiagen, Valencia, CA). DNA extraction was undertaken according to the protocol provided by the manufacturer, with the addition of desferroxamine to all solutions to a final concentration of 0.1 mM to protect against the oxidation of the DNA during the procedure.

Sample preparation for pooled-DNA adductome analysis

To grasp the overall picture of DNA adducts in the gastric mucosa of Japanese and Chinese patients, we performed a pooled-DNA adductome analysis. DNA

Table I. Clinicopathological profiles of the 22 cases from Lujiang (C) and Hamamatsu (J)

	Origin	Age	Sex (M: male; F: female)	Cancer location[a]	Histological type[b]
1	C	45	F	U	U
2	C	54	M	L	D
3	C	60	M	M	D
4	C	52	M	U	U
5	C	58	M	U	U
6	C	60	F	M	D
7	C	55	F	L	D
8	C	57	M	U	U
9	C	58	M	L	D
10	C	61	M	U	U
11	C	60	M	U	U
12	C	58	F	U	U
13	J	57	F	M	D
14	J	64	M	U	U
15	J	60	M	L	D
16	J	52	F	U	U
17	J	53	M	U	U
18	J	64	M	L	D
19	J	57	M	M	D
20	J	60	M	U	U
21	J	64	M	M	D
22	J	60	M	M	D

[a]U, M and L represent the upper, middle and lower thirds of the stomach.
[b]D, differentiated type (intestinal type); U, undifferentiated type (diffuse type).

samples from each patient (approximately 100 µg) were mixed with 54 µl of digestion buffer (17 mM sodium succinate and 8 mM calcium chloride, pH 6.0) containing 67.5 units of micrococcal nuclease (Worthington, Lakewood, NJ) and 0.255 units of spleen phosphodiesterase (Worthington). After 3 h of incubation at 37°C, three units of alkaline phosphatase (Sigma–Aldrich, St Louis, MO), 30 µl of 0.5 M Tris–HCl (pH 8.5), 15 µl of 20 mM zinc sulfate and 101 µl of milliQ water were added; the mixture was then incubated for another 3 h at 37°C. After incubation, an 87 µl aliquot from 10 Japanese samples (Patient Nos. 13–22) was taken and pooled as the Japanese group, and an 87 µl aliquot from 10 Chinese samples (Patient Nos. 2–11) was also pooled as the Chinese group. The pooled mixtures were concentrated to approximately 100 µl using a Speed-Vac concentrator, and 500 µl of methanol was added to precipitate the protein. After centrifugation, the methanol fraction (supernatant) was transferred to a new Eppendorf tube and evaporated to dryness, then redissolved in 320 µl of 30% dimethyl sulfoxide (5).

Sample preparation for quantification of lipid peroxidation-derived DNA adducts

Seven kinds of lipid peroxidation-derived DNA adducts [1,N^6-etheno-2′-deoxyadenosine (εdA), butanone-etheno-2′-deoxycytidine (BεdC), butanone-etheno-2′-deoxy-5-methylcytidine (BεmedC), butanone-etheno-2′-deoxyadenosine (BεdA), heptanone-etheno-2′-deoxycytidine (HεdC), heptanone-etheno-2′-deoxyguanosine (HεdG) and heptanone-etheno-2′-deoxyadenosine (HεdA)] were quantified basically as described by Chou and colleagues (6). A 58 µl aliquot of DNA digest was spiked with 2.2 µl of 4 nM stable-isotope internal standard mix ([$^{15}N_5$]-derivatives of εdA, BεdC, BεdA, HεdC, HεdG and HεdA), and the volume was reduced using a Speed-Vac concentrator; then, 100 µl of methanol was added to precipitate the protein. The methanol fraction (supernatant) was transferred to a new Eppendorf tube and evaporated to dryness, and then redissolved in 22 µl of 30% dimethyl sulfoxide.

DNA adductome analysis

For the DNA adducts analysis, a Quattro Ultima Pt triple-stage quadrupole mass spectrometer equipped with an Alliance 2695 separation module and a 2487 Dual λ Absorbance Detector (Waters, Milford, MA) was used. The pooled-DNA adductome analysis was performed in a manner similar to that described by Kanaly and colleagues (5). An aliquot of the digested DNA sample (20 µl) was injected and separated using a Shim-pack XR-ODS column (3.0 × 75 mm; Shimadzu, Kyoto, Japan). The column was eluted in a linear gradient of 5–80% methanol in water from 0 to 20 min at a flow rate of 0.2 ml/min. Multireaction monitoring was performed in the positive-ion mode. The experimental conditions were set as follows: ion source temperature, 130°C; desolvation temperature, 380°C; cone voltage, 35 V; collision energy, 15 eV; desolvation gas flow rate, 700 l/h; cone gas flow rate, 35 l/h and collision gas, argon. This strategy was designed to detect the neutral loss of 2′-deoxyribose from positively ionized 2′-deoxynucleoside adducts by monitoring the samples with $[M + H]^+ \rightarrow [M + H\text{-}116]^+$ transitions. Each of the Japanese- and Chinese-pooled samples were injected 15 times to complete the monitoring of the 451 multireaction monitoring transitions over the *m/z* range from the transition *m/z* 250→134 to the transition 702→586. The transitions of normal deoxynucleosides, including 252→136 ($[dA + H]^+$) and 268→152 ($[dG + H]^+$), were not monitored in the adductome analysis.

DNA adduct quantification

The same liquid chromatography and double mass spectrometry system was used for DNA adduct quantification. An aliquot (20 µl) of each sample was injected and separated using the Shim-pack XR-ODS column, eluted in a linear gradient of 5–30% methanol in water from 0 to 27 min and then of 30–80% methanol from 27 to 35 min, then kept in 80% methanol from 35 to 40 min at a flow rate of 0.2 ml/min. The collision energies and characteristic reactions monitored for the different DNA adducts were as follows [cone voltage (V), collision energy (eV), base ion→product ion]: [U-$^{15}N_5$]-εdA (35, 14, 280.9→164.9), [U-$^{15}N_3$]-HεdC (35, 10, 367.0→251.0), [U-$^{15}N_5$]-HεdA (35, 10, 393.0→277.0), [U-$^{15}N_5$]-HεdG (35, 10, 409.0→293.0), [U-$^{15}N_3$]-BεdC (35, 10, 324.8→208.6), [U-$^{15}N_5$]-BεdA (35, 10, 351.0→234.8), εdA (35, 14, 275.9→159.9), HεdC (35, 10, 364.0→248.0), HεdA (35, 10, 388.0→272.0), HεdG (35, 10, 404.0→288.0), BεdC (35, 10, 321.8→205.6), BεmedC (35, 20, 335.9→220.0) and BεdA (35, 10, 351.0→234.8). The amount of each DNA adduct was quantified by calculating the peak area ratio of the target DNA adduct and its specific internal standard ([U-$^{15}N_3$]-BεdC was used for BεdC and BεmedC). Calibration curves were obtained using authentic standards spiked with isotope internal standards.

Histological analysis

The tissues next to the sampled portion were used for histological evaluation using hematoxylin and eosin staining following the usual formalin-fixation and paraffin-embedding steps.

Detection of H.pylori DNA in the DNA sample

DNA from each sample was tested for *H.pylori* DNA according to a previously reported method, in principle (18,19). An automatic gene analyzing system, GENECUBE™ (Toyobo, Osaka, Japan), was used to detect S (sensitive to Clarithromycin) and R (resistant to Clarithromycin) DNA fragments encoding *H.pylori* 23S ribosomal RNA with an internal control, *CYP2C19*, for the human genome (20). All the cases had a peak at *CYP2C19* for either genotype. Cases without an R or S peak were considered to be *H.pylori* negative.

Statistical analysis

For each adduct level, a Wilcoxon rank sum test was performed to compare the DNA adduct numbers/10^9 bases. We noticed one outlier case in our data set, and a cluster analysis validated the independency of this single case. After an exclusion of the outlier case, a discriminant function analysis was performed using seven adduct levels to categorize the cases based on the origin of tissue (Lujiang or Hamamatsu), the location of the cancer (upper, middle or lower part of the stomach), the histology of the cancer (differentiated or undifferentiated) and the sex (male or female). All the calculations were performed using the statistics package software JMP7™ (SAS Institute Japan Ltd, Tokyo, Japan).

Institutional review boards

The study protocol was approved by the institutional review boards of Hamamatsu University School of Medicine (23–91), Lujiang People's Hospital and Nanjing University.

Results

DNA adductome

A DNA adductome map of Japanese and Chinese gastric mucosa DNA is shown in Figure 1. This adductome map corresponds to a mixture of 10 Japanese samples (blue) and a mixture of 10 Chinese samples (red), so that this map reflects the average picture of the gastric mucosa in both countries. In this adductome map, peaks that might be derived from normal deoxynucleosides were omitted. We identified 141 peaks in the Japanese sample and 159 peaks in the Chinese sample. Ninety-two of these peaks were present in both the Japanese and Chinese samples. Although most of the peaks (circles) shown on this map have not been identified, we found peaks corresponding to εdA (m/z: 276, RT 12.5 min) and HεdC (m/z: 364, RT 22.8 min), which are lipid peroxidation-derived DNA adducts. These observations motivated

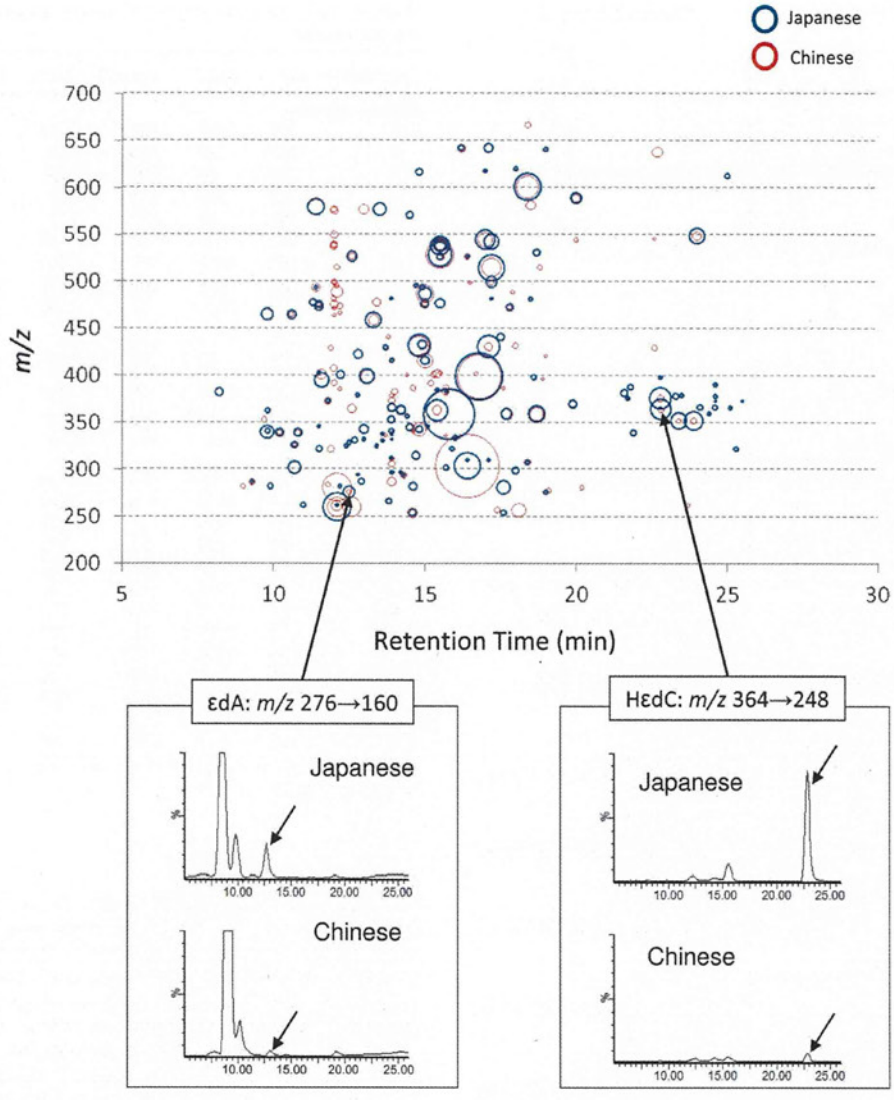

Fig. 1. Pooled-DNA adductome map of gastric mucosa samples from 10 Japanese and 10 Chinese patients. In this plot, the bubble size represents the relative peak area of MS/MS chromatogram. The liquid chromatography and double mass spectrometry peaks corresponding to the lipid peroxidation-induced DNA adducts, εdA and HεdC, are shown.

us to quantify specific lipid peroxidation-derived DNA adducts in individual samples.

Quantification of lipid peroxidation-derived DNA adducts

The representative chromatogram from one individual is shown in Figure 2. Chromatogram of 1 nM standards (left panel) and a stomach sample (right panel) were shown. The numbers of seven kinds of lipid peroxidation-derived DNA adducts per 10^9 bases in 22 cases of gastric mucosa are shown in Table II. The approximate detection limits for each DNA adduct were 1.7 per 10^9 bases (εdA, BεdC, BεmedC and BεdA), 3.3 per 10^9 bases (HεdC) and 16.5 per 10^9 bases (HεdA and HεdG). Among the seven adducts, εdA and HεdC were detected in all 22 cases, and their levels were extremely high, compared with previous reports describing their presence in other organs (6). Actually, the median value for εdA in the Chinese and Japanese samples was 3–5 adducts per 10^7 bases, and the median value for HεdC was 5–13 adducts per 10^7 bases. These values (3–13 adducts per 10^7 bases) are as high as the level of 8-hydroxydeoxyguanosine, one of the most prevalent DNA adducts currently known (6,21). Because minimal comparable data for the levels of these adducts in human gastric tissues is available in previous reports, we can only report the presently observed values. In addition, some DNA adducts other than εdA and HεdC were also detected at a high frequency, and their levels were also high compared with their levels in previous reports describing their presence in other organs (6,22). The median values for the DNA adducts were higher in the Japanese samples than in the Chinese samples. Three adducts, BεdC, BεdA and HεdA, were significantly higher in the Japanese gastric mucosa (Wilcoxon rank sum test) (Figure 3).

Cluster analysis and discriminant function analysis

A cluster analysis using the Ward method was undertaken (23). The dendrogram (Figure 4) identified one outlier case (case number 14) as a single isolated group (green). Case number 14 had extremely high values for almost all the adducts that were investigated.

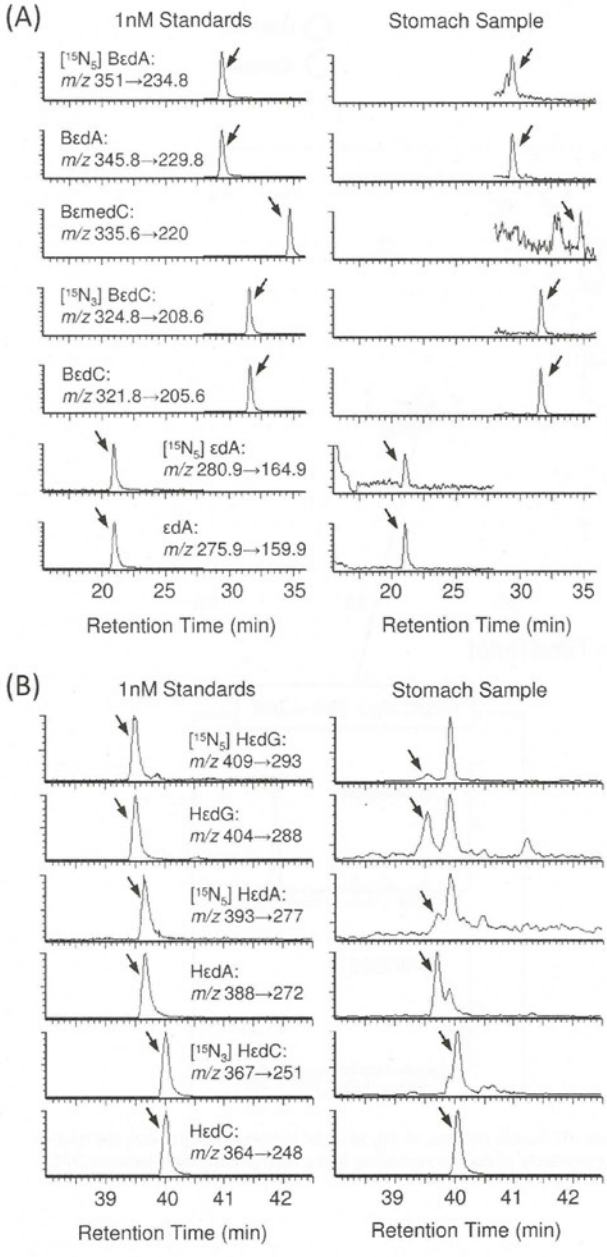

Fig. 2. Representative liquid chromatography and double mass spectrometry chromatogram of the lipid peroxidation-induced DNA adducts. Chromatogram of 1 nM standards (left panel) and a stomach sample (right panel) were shown. This sample is from the case number 21 of Table II. (**A**) Chromatogram of the εdA, BεdC, BεmedC and BεdA with their stable isotopes. (**B**) Chromatogram of the HεdC, HεdA and HεdG with their stable isotopes.

Because we were interested in whether the profiles of these seven adduct levels could identify the origin of the mucosa, we performed a discriminant function analysis. Excluding the outlier case, the discriminant function analysis generated a discriminant score in which the threshold of each category was set to 20%. When the origin of the tissue, Lujiang or Hamamatsu, was used as the dependent category, we were able to discriminate the origins of the tissues perfectly according to the seven adduct levels (Table III). The results of the discriminant function analysis were successfully transformed into a plot graph against two covariants (Figure 5).

Table II. DNA adduct level (per 10^9 bases) in Japanese and Chinese gastric mucosa samples

Case number	εdA	BεdC	BmεdC	BεdA	HεdC	HεdA	HεdG
Chinese samples							
1	281	447	ND	91	1619	305	118
2	211	34	ND	ND	112	ND	ND
3	797	73	ND	ND	149	ND	47
4	333	248	ND	ND	807	353	53
5	209	34	ND	ND	106	33	19
6	158	438	14	61	846	178	17
7	1792	1649	ND	319	3943	1491	209
8	1434	983	ND	220	3390	642	202
9	1105	1071	18	106	1662	331	108
10	190	ND	ND	ND	35	ND	ND
11	383	16	ND	ND	57	ND	ND
12	119	17	ND	ND	37	ND	18
Maximum	1792	1649	18	319	3943	1491	209
Median	307	160.5	ND	ND	478	105.5	33
Minimum	119	ND	ND	ND	35	ND	ND
Japanese samples							
13	262	94	ND	66	436	371	25
14	2763	7616	444	2261	29298	34456	482
15	627	989	ND	231	1630	594	50
16	505	697	23	206	1575	672	69
17	406	1204	ND	220	1103	266	ND
18	436	294	ND	82	869	482	73
19	763	468	ND	107	1022	427	33
20	571	1508	24	383	2523	974	110
21	964	2261	57	472	3035	1520	190
22	390	91	ND	25	203	106	ND
Maximum	2763	7616	444	2261	29298	34456	482
Median	538	843	ND	213	1339	538	59.5
Minimum	262	91	ND	25	203	106	ND

ND, not detectable.

Clinicopathological analysis

H.pylori DNA was detected in eight cases: four in Chinese gastric mucosa samples and four in Japanese gastric mucosa samples. On the other hand, genotyping for 2C19 was successful in all the cases; thus, our detection system for *H.pylori* DNA seemed sound. No differences in any of the adduct levels or in the profile of the seven adducts was seen between the *H.pylori* DNA-positive and -negative cases (data not shown).

Clinicopathological parameters including sex, age, location of cancer and histological type were compared with the adducts levels, but no correlations were obtained (data not shown). A determinant function analysis based on the parameters of sex, age, location of cancer and histological type did not generate a discriminant function capable of dividing these categories (data not shown).

Discussion

We identified lipid peroxidation-derived DNA adducts in the human stomach for the first time. Considerable numbers of seven different adducts were demonstrated. In our previous study, we quantified these DNA adducts in 68 Japanese autopsy tissues from various organs other than the stomach (lung, colon, pancreas, spleen, liver, kidney and small intestine (6)). The median values of the lipid peroxidation-derived DNA adducts in Japanese gastric mucosa observed in this study were one order of magnitude higher than those observed in the other organs. The active inflammation in the stomach may be one of the most plausible explanations for this phenomenon, but complete information on the other adducts must also be considered.

Differences in these adduct levels between the Chinese and Japanese gastric mucosa samples were not, at first glance, apparent, but a discriminant analysis disclosed detectable differences according to the origin of the gastric mucosa. When examined separately, three of the seven adducts (BεdC, BεdA and HεdA) were

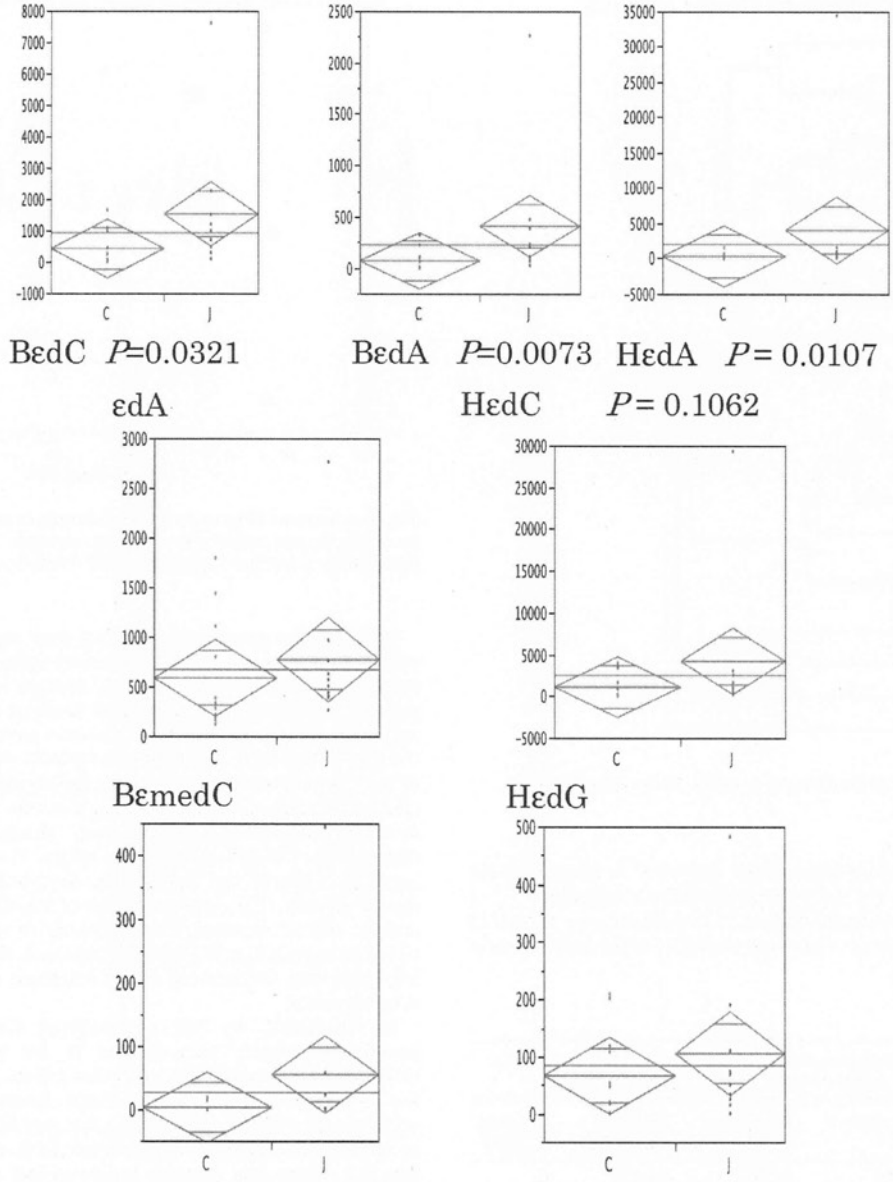

Fig. 3. Comparison of each adduct level between the Lujiang (C) and Hamamatsu (J) cases. The levels of BεdC, BεdA and HεdA were significantly higher for the Hamamatsu cases ($P < 0.05$ according to a Wilcoxon rank sum test). The horizontal lines in the diamonds indicate the upper quadrant, the mean and the lower quadrant. The axis indicates the numbers of adducts per bases.

significantly higher in the Japanese stomach specimens than in the Chinese stomach specimens. The significance of this observation remains unknown.

Lujiang county is known as an area of China where gastric cancer is endemic (24); thus, we expected higher adduct levels in this group than in the Japanese group. This analysis, however, showed that the Japanese stomach mucosa samples had higher levels of these lipid peroxidation-derived adducts. This observation may reflect the fact that the presently evaluated markers were limited to inflammation-related markers. Actually, our adductome map (Figure 1, (5,6)) for these specimens had many other un-annotated spots, some of which were more prevalent in the Chinese stomach specimens.

In our previous papers, Chou and colleagues argued that the adducts investigated in this study are formed by exposure to 4-ONE [4-oxo-2(*E*)-nonenal] and 4-OHE [4-oxo-2(*E*)-hexenal], which can be made from ω-6 and ω-3 polyunsaturated fatty acids endogenously. They cited a paper by Blair's group and hypothesized that COX2 and lipoxygenase contributed to the formation of these adducts (25). On the other hand, continued inflammation, or chronic atrophic gastritis, has long been thought to predispose an individual to gastric cancer (26,27). Hence, our evidence of the presence of oxidation-related DNA adducts may strengthen the idea of the inflammation-mediated pathogenesis of gastric cancer (11).

We showed the usefulness of the adductome profile for indicating the origin of a specimen, but whether these adducts actually indicate a gastric cancer predisposition warrants the further investigation of gastric mucosa samples from subjects without gastric cancer. Previously, inflammation-mediated carcinogenesis has been discussed in the context of dynamic changes of cellular machinery such

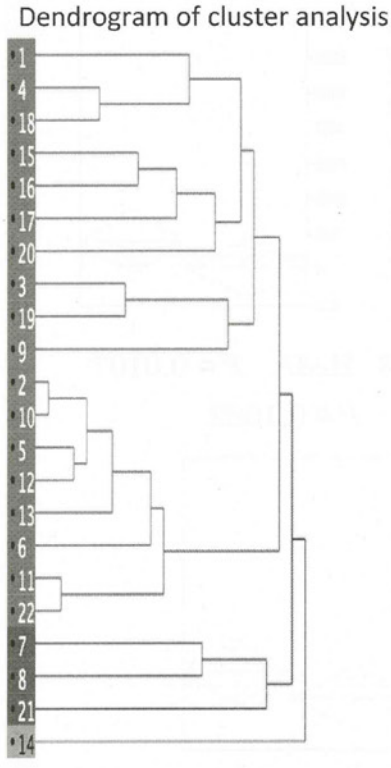

Fig. 4. Cluster analysis of the 22 cases. Case No. 14 was identified as an outlier.

as microRNA, not of formation of DNA adducts (28), but some of the adducts described in this study are known to be mutagenic (29–31); thus, these lipid peroxidation-derived DNA adducts may, at least in part, be responsible for the carcinogenic origins of the human gastric cancers in this series.

Table III. Results of discriminate analysis using seven adduct levels as the discriminating score to predict the origin of the specimen in 21 cases

Real origin	Square distance	Probability	Predicted probability	Predicted category
C	8.91653	0.9986	0.9986	C
C	1.41919	0.8686	0.8686	C
C	8.60098	0.9444	0.9444	C
C	6.09944	0.998	0.998	C
C	1.36231	0.9175	0.9175	C
C	7.82509	0.9769	0.9769	C
C	14.87972	0.6381	0.6381	C
C	12.39559	0.9982	0.9982	C
C	11.20971	0.9999	0.9999	C
C	1.71078	0.7945	0.7945	C
C	2.57192	0.7579	0.7579	C
C	2.10673	0.9117	0.9117	C
J	2.73173	0.947	0.947	J
J	2.77308	0.9958	0.9958	J
J	5.78756	0.9818	0.9818	J
J	11.33611	0.9832	0.9832	J
J	5.8705	0.5033	0.5033	J
J	3.35645	0.8798	0.8798	J
J	5.57043	0.9998	0.9998	J
J	13.27304	0.9983	0.9983	J
J	3.2031	0.575	0.575	J

C indicates Lujiang Hospital and J indicates Hamamatsu University Hospital.

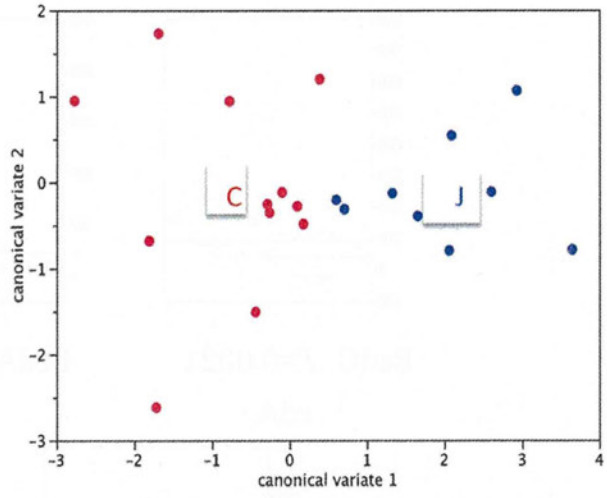

Fig. 5. A scattered diagram showing a discriminant analysis of the 21 cases (excluding the one outlier case) with seven variables. The red and blue dots represent the cases from Lujiang (C) and Hamamatsu (J), respectively.

From another perspective, our data may suggest that a pathway other than the peroxidation-inflammatory pathway may contribute to carcinogenesis. The Chinese gastric mucosa samples derived from patients in Lujiang county had lower levels of oxidative DNA damage, but Lujiang has a higher prevalence of gastric cancer than Japan. Other environmental insults may be revealed with further annotation of the observed adducts, possibly including alkylating agent-related adducts. Actually, the pooled-DNA adductome map, which was used as a screening procedure in this study, contains many other peaks that may not be lipid peroxidation related. It is assumed that there are many subjects who do not have detectable lipid peroxidation-related adducts. The continued effort of identifications of the other adducts will be necessary to comprehensive understanding of gastric carcinogenesis, and adductome approach, though at the burgeoning stage, may become one of the important omics in the field of carcinogenesis.

In conclusion, we first demonstrated the existence of lipid peroxidation-related DNA adducts in the human stomach and addressed their implications in the assessment of the environmental and endogenous exposure of human beings to these possible mutagens. In addition, considering that gastric cells have a battery of repair genes that respond to or repair DNA damage, the presently reported results may promote understanding of the role of repair genes in gastric carcinogenesis, a topic that has recently attracted enthusiastic interest in the field of carcinogenesis research (32–36).

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Conflict of Interest Statement: None declared.

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

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

Carcinogenesis

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

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

Key words: gastric cancer, *Helicobacter pylori*, Mongolian gerbil 1-nitrosoindole-3-acetonitrile, indole-3-acetonitrile

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

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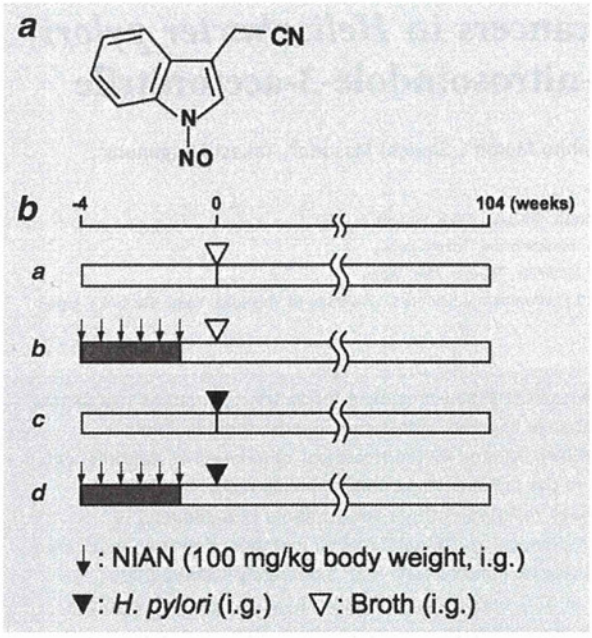

Figure 1. Chemical structure of NIAN and experimental protocol for the carcinogenicity study. (*a*) Chemical structure of NIAN. (*b*) Male 6-week-old MGs were orally administered NIAN (100 mg/kg) in 50% DMSO (groups B and D) or 50% DMSO alone (groups A and C) two times a week for 3 weeks. One week after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

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

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

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

Material and Methods

Materials

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

Preparation of NIAN

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

Bacterial culture

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

Animal treatment

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

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

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

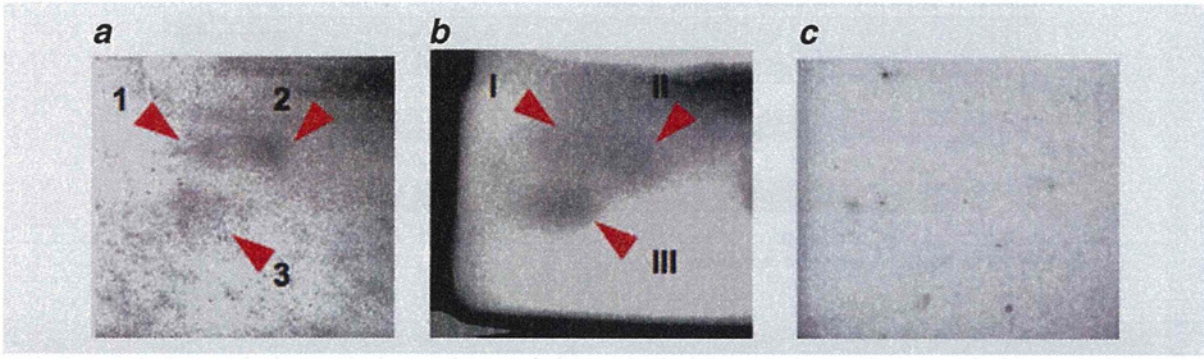

Figure 2. Autoradiograms of NIAN-DNA adducts in glandular stomach of MGs or calf thymus DNA treated with NIAN. Adducts were analyzed by ^{32}P-postlabeling method, as described in the Material and Methods. DNA samples were isolated from glandular stomach of MGs (*a*) or calf thymus DNA (*b*) after treatment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

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

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

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

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

Histological examination

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

Statistical analysis

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

Results

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

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

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

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

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

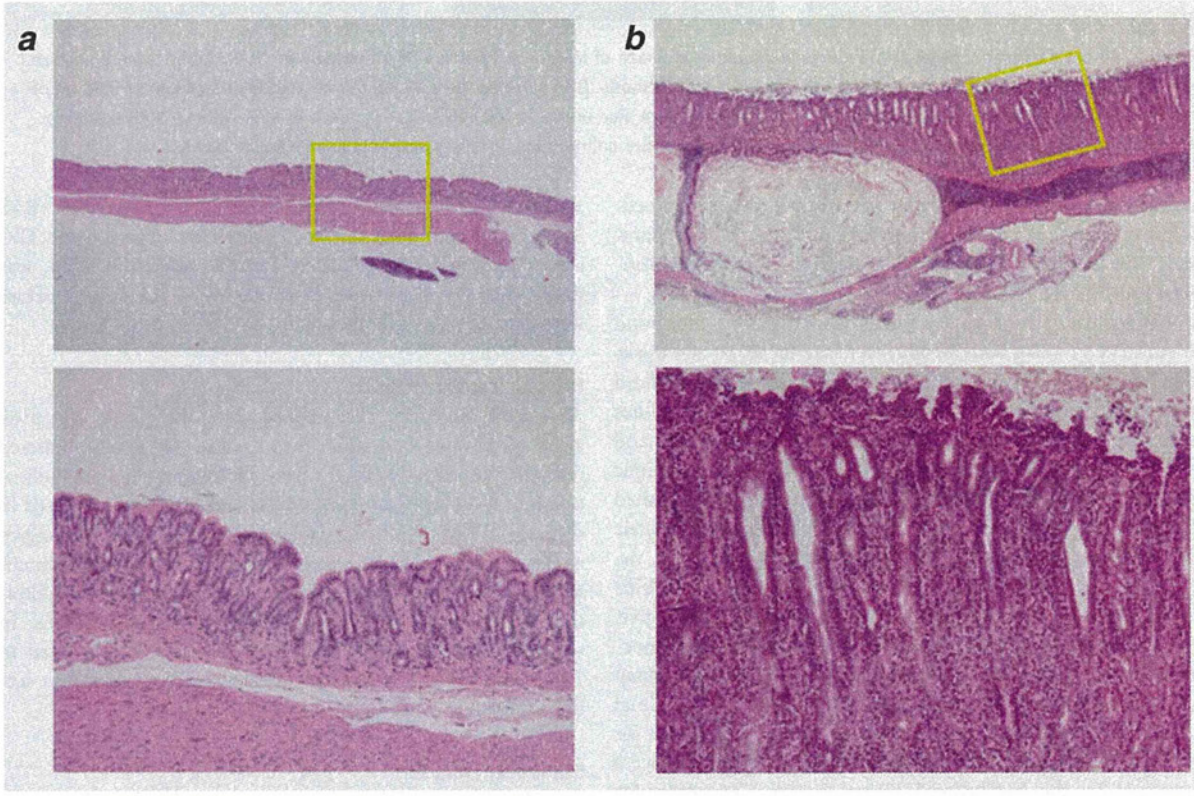

Figure 3. Macroscopic and microscopic views of gastritis in MGs infected or uninfected with *H. pylori*. (*a*) Normal gastric mucosa in group A. (*b*) Severe infiltration of many inflammatory cells with development of heterophilic proliferative glands in group C; H&E staining, ×40. Yellow boxes are shown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

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

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

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

Microscopically, gastritis, featuring infiltration of many inflammatory cells, and hyperplastic change of glandular epithelium, and erosion were observed in the pyloric regions of

Table 2. Incidence of glandular stomach adenocarcinoma in MGs

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

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

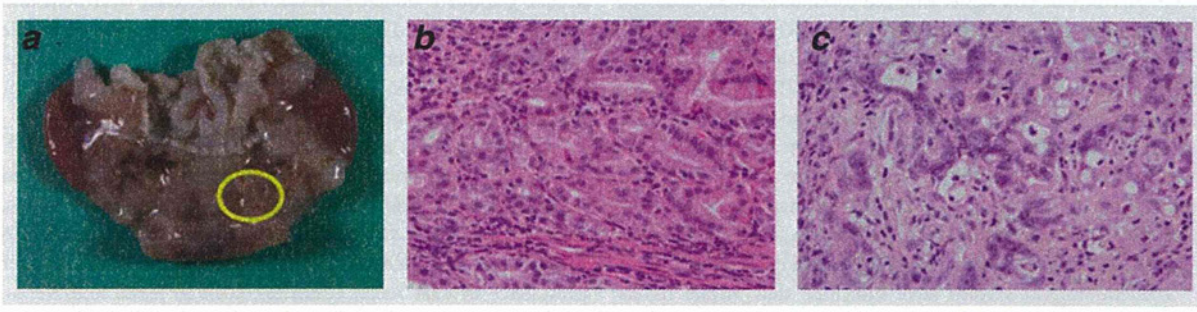

Figure 4. Histological findings of gastric adenocarcinoma in the animals treated with both NIAN and *H. pylori*. (*a*) Typical macrograph of a stomach. The yellow circle shows the suspected lesion of gastric cancer. (*b*) Well differentiated adenocarcinoma. (*c*) Moderately differentiated adenocarcinoma.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

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

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

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

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

Discussion

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

Glandular stomach adenocarcinomas induced by NIAN treatment plus *H. pylori* infection were located in the pyloric region, similar to MNNG or MNU treatment plus *H. pylori* infection-induced glandular stomach adenocarcinomas in MGs.[26,27] Meanwhile, no glandular stomach cancers were observed in the groups of *H. pylori*-infected MGs without NIAN treatment, which is consistent with previous studies,[26,27] nor in the group treated with only NIAN. These findings indicated that *H. pylori* is a strong promoter of gastric carcinogenesis. Histological examination revealed that the tumors developed by NIAN + *H. pylori* were of well or moderately differentiated adenocarcinomas. Well or poorly differentiated adenocarcinomas and signet ring cell carcinomas were observed in *H. pylori*-infected MGs treated with MNNG or MNU.[26,27] Further studies are required to clarify the histological variety of stomach adenocarcinomas induced by NIAN, MNNG or MNU, since the type of cancer might depend on the genotoxic action of chemical carcinogens, rather than the effects of *H. pylori* infection.[27] In addition, tumors were observed in skin and kidney, which were suspected to spontaneously develop. The MGs have been reported to develop spontaneous skin tumors such as sebaceous and squamous cell carcinoma.[34]

Epidemiological studies have indicated that nitrate intake increases gastric cancer risk, and major sources are vegetables including Chinese cabbage, spinach and parsley.[14] Indole-3-acetonitrile, a precursor of NIAN, is distributed widely in cruciferous vegetables including Chinese cabbage and sprouts.[35] Furthermore, fava beans (*Vicia faba*), which are commonly consumed in Colombia, give rise to a potent mutagen in the presence of nitrite under acidic conditions.[36] The nitrosatable precursor of the mutagen in fava beans and the major product of nitrosation are reported to be an indole compound, 4-chloro-6-methoxyindole and an *N*-nitroso compound, 4-chloro-2-hydroxy-N^1-nitroso-indolin-3-one oxime, respectively.[37] Other indole compounds are also reported to produce direct-acting mutagens after nitrite treatment under acidic conditions.[38,39] In general, conversion of indole derivatives to nitrosated forms *in vitro* is known to be rapid and efficient at physiologically feasible nitrite concentrations with the low pH of the human stomach.[37] Thus, it is conceivable that nitrosation of indole compounds such as indole-3-acetonitrile probably occurs in human stomach. On the other hand, nitric oxide is suggested to be produced by activated macrophages in inflamed organs with *H. pylori* infection.[18] Therefore, nitrosation of indole compounds could be mediated by both acid catalysis and inflammatory responses in the human stomach.[18,20,37–40] On the basis of the conversion rate of NIAN from indole-3-acetonitrile under physiological conditions, the dose of NIAN used in the present study appears about 500–1000 fold the expected human exposure to NIAN *via* fresh or pickled Chinese cabbage. However, humans continually consume various kinds of foods containing indole compounds and nitrate during ordinary life. Thus, it is probable that the total amount of nitroso-indole compounds would be much closer to the dose of NIAN used in the present study. Moreover, it has been reported that low doses of chemical carcinogens, such as MNNG and MNU, could induce glandular stomach cancers in rodents under inflammation conditions including NaCl treatment and *H. pylori* infection, but hardly induce glandular stomach cancer without NaCl treatment and *H. pylori* infection. Therefore, the continuous intake of indole compounds and nitrate may play an important role for gastric carcinogenesis in East Asian countries still with a high salt consumption and *H. pylori* infection rate.

Gastric cancer is tending to decline in most countries.[41–43] One of the explanations for this tendency is the reduced prevalence of *H. pylori* infection.[42] Changes in dietary habits, mainly being lower salt consumption, could be also related to reduced gastric cancer incidence. However, the gastric cancer prevalence in East Asian countries, such as Japan and Korea, is still high.[2] At present, we have not succeeded in detecting NIAN in human bodies nor the exposure levels of the precursor, indole compounds for humans. Thus, it is necessary to estimate the human exposure levels to nitroso-indole compounds including NIAN, and to study further animal experiments and epidemiological analyses for clarification of contribution of nitroso-indole compounds under *H. pylori* infection in humans gastric carcinogenesis.

In conclusion, the present study demonstrated that NIAN can induce gastric cancer in *H. pylori*-infected MGs. It is noteworthy that nitrosatable precursors widely exist in foods. Thus, it is suggested that *N*-nitroso indole compounds including NIAN might contribute to the frequent development of gastric cancer in East Asian countries such as Japan and Korea in which the prevalence of *H. pylori* infection is relatively high. Further studies of interaction with other dietary elements appear warranted to promote the prevention of human gastric cancer.

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