Lipid peroxidation-induced DNA adducts in human gastric mucosa

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DNA adducts are a major cause of DNA mutation and DNA muta-
tion-related diseases, but the simultaneous identification of multi-
ple 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 comprehen-
sive 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 (BedC), butanone-etheno-2′-deoxy-5-methylcytidine, butanone-etheno-2′-deoxyadenosine (BedA), heptanone-etheno-2′-deoxycytidine, heptanone-etheno-2′-deoxyadenosine (HeDA) 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 10⁴ bases. Although the presence of Helicobacter pylori DNA in the mucosa was not related to these adduct levels, the levels of BedC, BedA and HeDA 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 investiga-
tion 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 informa-
tion on the susceptibility to and the initiation of cancer in particular organs and particular individuals (2).

Abbreviations: BrdA, butanone-etheno-2′-deoxyadenosine; BrdC, butanone-etheno-2′-deoxycytidine; BrmC, butanone-etheno-2′-deoxy-5-methylcytidine; rChdA, 1,N6-etheno-2′-deoxyadenosine; rChdC, heptanone-etheno-2′-deoxyadenosine; rChdC, butanone-etheno-2′-deoxyguanosine.

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 adduc-
tome 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 the development of gastric cancer (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 P³2-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 P³2-postlabeling method cannot further delineate the molecular species in DNA from the gastric mucosa. Recently, our group established a method consisting of liquid chromatography fol-
lowed by double tandem mass spectrometry to identify numerous adducts in human and animal tissues (4–6,16) during a single experi-
ment. In contrast with the P³2-postlabeling method, this method di-
rectly 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-caus-
ing 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 stom-
ach samples obtained during gastrectomies for the resection of gastric cancer and validated seven different lipid peroxidation-derived DNA adducts in the human stomach for the first time.

Materials and methods

DNA extraction from gastric mucosa

DNA 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, patholo-
gists 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 sam-
ple, 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

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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 Chinese 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\(^{\epsilon}\)-etheno-2′-deoxyadenosine (εdA), butanone-etheno-2′-deoxycytidine (BrdC), butanone-etheno-2′-deoxy-5-methylcytidine (BrdmC), butanone-etheno-2′-deoxyadenosine (BrdA), 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 4nM stable-isotope internal standard mix ([15N\(^{\epsilon}\)]-derivatives of εdA, BrdC, BrdA, 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 (5).

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 column emissions and characteristic reactions measured for the different DNA adducts were as follows [cone voltage (V), collision energy (eV), base ion—product ion]: [U-15N\(^{\epsilon}\)]-εdA (35, 14, 280.9→164.91), [U-15N\(^{\epsilon}\)]-HεdC (35, 10, 367.0→251.0), [U-15N\(^{\epsilon}\)]-HεdA (35, 10, 393.0→277.0), [U-15N\(^{\epsilon}\)]-HεdG (35, 10, 409.0→293.0), [U-15N\(^{\epsilon}\)]-BrdC (35, 10, 324.8→208.6), [U-15N\(^{\epsilon}\)]-BrdA (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), BrdC (35, 10, 321.8→205.6), BrmedC (35, 20, 335.9→220.0) and BrdA (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-15N\(^{\epsilon}\)]-BrdC was used for BrdC and BrmedC). 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 CY2P219 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\(^{6}\) 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 several odd 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 deoxynucleotides 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
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 10⁹ bases in 22 cases of gastric mucosa are shown in Table II. The approximate detection limits for each DNA adduct were 1.7 per 10⁹ bases ($\epsilon_dA$, $B\epsilon_dC$, $B\epsilon_medC$ and $B\epsilon_dA$), 3.3 per 10⁹ bases ($H\epsilon_dC$) and 16.5 per 10⁹ bases ($H\epsilon_dA$ and $H\epsilon_dG$). Among the seven adducts, $\epsilon_dA$ and $H\epsilon_dC$ 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 $\epsilon_dA$ in the Chinese and Japanese samples was 3–5 adducts per 10⁷ bases, and the median value for $H\epsilon_dC$ was 5–13 adducts per 10⁷ bases. These values (3–13 adducts per 10⁷ 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 $\epsilon_dA$ and $H\epsilon_dC$ 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, $B\epsilon_dC$, $B\epsilon_dA$ and $H\epsilon_dA$, 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.
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).

**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 adduct levels 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 (BrdC, BrdA and HrdA) were...
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 α-6 and α-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 as...
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.

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.

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

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<th>Real origin</th>
<th>Square distance</th>
<th>Probability</th>
<th>Predicted probability</th>
<th>Predicted category</th>
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</table>

C indicates Lujiang Hospital and J indicates Hamamatsu University Hospital.

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**Funding**

This project was approved by the government of Jiangsu Province, China, under the project title, ‘Study on the relationship between the epi-germline mutation of tumor suppressor genes and the pathogenesis of gastrointestinal cancers’ (the International Science and Technology Cooperation Project of Jiangsu, grant number: BZ2008055). This work was also supported by Grants-in-Aids (Research on International Cooperation in Medical Science, Grants-in-Aids for Cancer Research, 21-1) from the Ministry of Health, Labour and Welfare; the Japan Society for the Promotion of Science (22590356 and 22790378); the Ministry of Education, Culture, Sports, Science and Technology (221S0001); the Princess Takamatsu Cancer Research Foundation and the Smoking Research Foundation of Japan.

**Conflict of Interest Statement:** None declared.
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Received August 2, 2012; revised September 4, 2012; accepted October 7, 2012.