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CASE REPORT

Hepatocellular adenoma associated with familial adenomatous polyposis coli

Keisuke Inaba, Takanori Sakaguchi, Kiyotaka Kurachi, Hiroki Mori, Hong Tao, Toshio Nakamura, Yasuo Takehara, Satoshi Baba, Masato Maekawa, Haruhiko Sugimura, Hiroyuki Konno

Keisuke Inaba, Department of Surgery, Iwata City Hospital, Iwata 438-8500, Japan
Keisuke Inaba, Takanori Sakaguchi, Kiyotaka Kurachi, Toshio Nakamura, Hiroyuki Konno, Second Department of Surgery, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan
Hiroki Mori, Hong Tao, Satoshi Baba, Haruhiko Sugimura, Department of Pathology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan
Yasuo Takehara, Department of Radiology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan
Masato Maekawa, Department of Laboratory Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan
Author contributions: Inaba K and Sakaguchi T treated the patient and wrote the manuscript; Kurachi K and Nakamura T treated the patient and helped to draft the report; Takehara Y reviewed the radiological features of the case; Tao H and Maekawa M examined the genetic alterations; Mori H, Baba S and Sugimura H contributed to the pathological examination and decided the final pathological diagnosis; Konno H was responsible for the patient management and supervised and approved the final manuscript.
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Correspondence to: Keisuke Inaba, MD, Department of Surgery, Iwata City Hospital, 512-3 Okubo, Iwata 438-8500, Japan. kinaba@hospital.iwata.shizuoka.jp
Telephone: +81-538-385000 Fax: +81-538-385050
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Abstract

Hepatocellular adenoma (HCA) is a benign liver tumor that most frequently occurs in young women using oral contraceptives. We report a rare case of HCA in a 29 years old female with familial adenomatous polyposis (FAP). The first proband was her sister, who underwent a total colectomy and was genetically diagnosed as FAP. A tumor, 3.0 cm in diameter, was detected in the right lobe of the liver during a screening study for FAP. A colonoscopy and gastroendoscopy revealed numerous adenomatous polyps without carcinoma. The patient underwent a total colectomy and ileo-anal anastomosis and hepatic posterior sectoriectomy. The pathological findings of the liver tumor were compatible with HCA. The resected specimen of the colon revealed multiple colonic adenomatous polyps. Examination of genetic alteration revealed a germ-line mutation of the adenomatous polyposis coli (*APC*) gene. Inactivation of the second *APC* allele was not found. Other genetic alterations in the *hepatocyte nuclear factor 1 alpha* and *β-catenin* gene, which are reported to be associated with HCA, were not detected. Although FAP is reported to be complicated with various neoplasias in extracolic organs, only six cases of HCA associated with FAP, including the present case, have been reported. Additional reports will establish the precise mechanisms of HCA development in FAP patients.

Key words: Hepatic adenoma; Familial adenomatous polyposis coli; Extrahepatic manifestation; Adenomatous polyposis coli gene; Hepatocyte nuclear factor 1 alpha

Peer reviewer: Tsung-Jung Lin, MD, Department of Gastroenterology, Ren-Ai Branch, Taipei City Hospital, 11 F, No. 10, Sec. 4, Renai Rd., Da-an District, Taipei 106, Taiwan, China

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INTRODUCTION

Hepatocellular adenoma (HCA) is a benign liver tumor that usually arises in women who are over 30 years old and have used oral contraceptives for over 5 years[1]. Other risk factors associated with HCA have been described, including glycogen-storage diseases, androgens, anabolic steroids, diabetes mellitus, some drugs and pregnancy[2-5].

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease caused by a mutation in the adenomatous polyposis coli (*APC*) gene. FAP is characterized by the early onset of multiple colorectal adenomatous polyps, with an inevitable progression to carcinoma if left untreated. Additionally, FAP is known to be associated with extracolic neoplasms in various other organs; adenomas and carcinomas of the upper gastrointestinal tract, desmoid tumors and thyroid carcinomas[6]. Due to familial screening and prophylactic colectomies, the prognosis of FAP patients has improved[6-8]. Thus, extracolic tumors have become more important causes of mortality[7]. Duodenal or periampullary cancer and desmoids are the two main causes of mortality after a total colectomy[9]. Other rare extracolic manifestations include cancers of the thyroid, liver, bile ducts and central nervous system[6,7,9]. HCA is rare for FAP-associated extracolic neoplasms[10].

Herein, we report a rare case of HCA concomitant with FAP. She had no history of oral contraceptive use or other risk factors for HCA. We summarize previous case reports[5,10-14] and consider HCA arising in FAP patients.

CASE REPORT

A 29 years old Japanese woman was called for familial surveillance of FAP because her 27 years old sister had undergone a total colectomy due to the diagnosis of ascending colon cancer arising from FAP, already confirmed by gene analysis. Her 46 years old father died of gastric cancer but FAP was uncertain. Her son had suffered from hepatoblastoma which had been resected when he was 18 mo old. Her preoperative clinical laboratory tests, including liver function, were normal. Serologically, serum hepatitis B and hepatitis C virus markers were negative. Serum levels of alpha-fetoprotein and des-γ-carboxy prothrombin were also within normal ranges. Preoperative computed tomography (CT) showed a tumor in the posterior sector of the right lobe, measuring 28 mm in diameter. The tumor showed a slight inhomogeneous low density area on the unenhanced scan when compared with the surrounding liver parenchyma (Figure 1). The tumor was well enhanced in the early phase after the contrast medium injection. The tumor became indistinguishable in the late phase. Although the tumor was not detectable on T1-weighted magnetic resonance imaging (MRI), it was detected as a mild hyper-intense tumor in the posterior sector on T2-weighted MRI (Figure 1). The tumor was indiscernible in the arterial phase, but became a hypo-intense area in the hepato-biliary phase after gadolinium ethoxybenzyl diethylenetriaminepentaacetic acid enhancement on T1-weighted MRI. No obvious

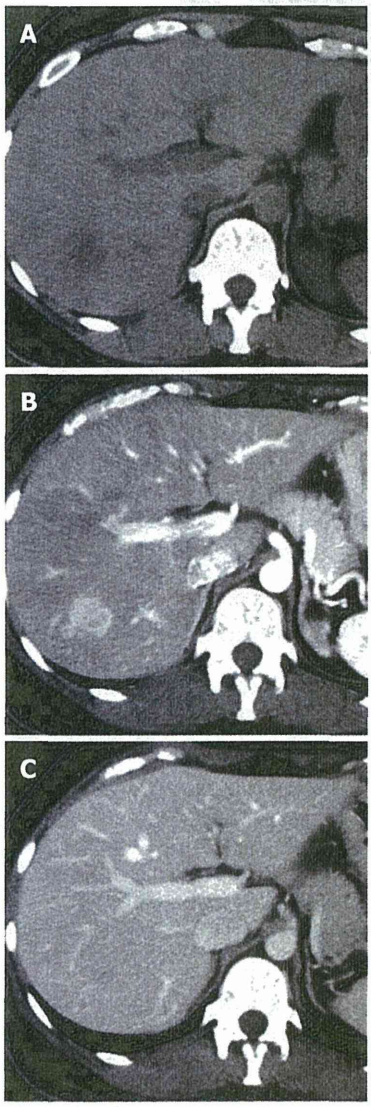

Figure 1 Preoperative computed tomography scan revealed a 28 mm tumor in the posterior sector of the right hepatic lobe. A: Plain computed tomography showed a tumor as a slight low density area; B: The tumor was inhomogeneously enhanced with a ragged border during the early phase; C: The tumor was indistinguishable in the late phase.

capsular formation or visible central scars were observed (Figure 2). Hepatic arteriography showed a tumor stain without any abnormalities in vascular structure or angioplany. A total colonoscopy revealed numerous polyps of various sizes throughout the colon and rectum but no obvious colorectal carcinoma was found. Gastroendoscopy also found thick polyps without carcinoma.

On the basis of clinical features[15] and previous literature[10-13], we performed a total colectomy and ileoanal anastomosis and hepatic posterior sectoriectomy on December 2008. Macroscopically, numerous polyps of various sizes, including one lateral spreading tumor in the ascending colon, were found in the mucosal surface of the resected colon specimen. In the cut surface of the resected liver specimen, the tumor grossly showed a faint yellow tumor without hemorrhage or necrosis. The

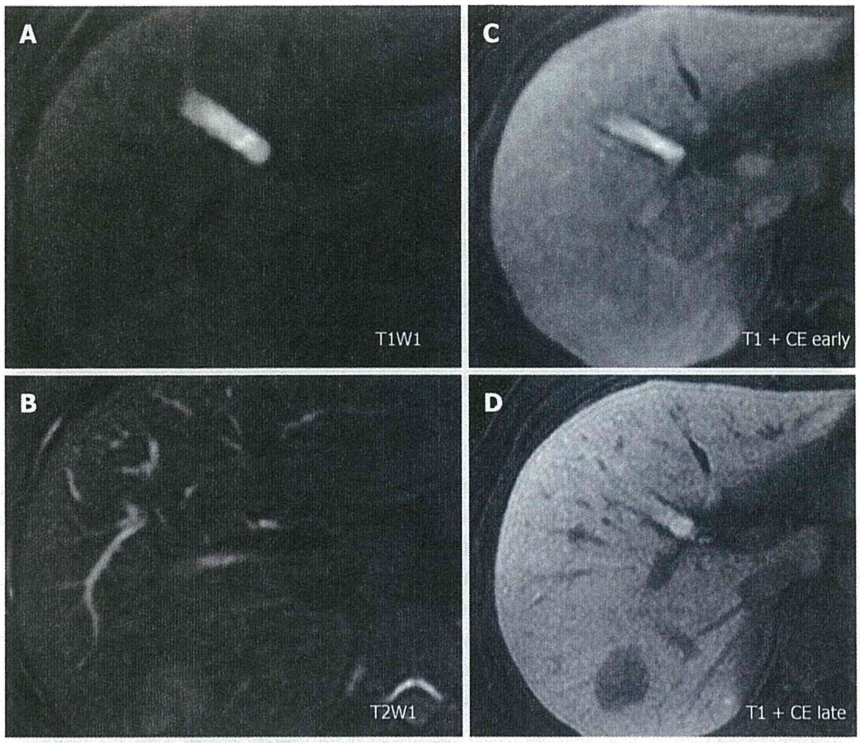

Figure 2 Magnetic resonance imaging of the tumor. A: The tumor showed an iso-intensity with the surrounding liver parenchyma on T1-weighted imaging; B: The tumor was visualized as a heterogeneous hyper-intense mass on T2-weighted imaging; C and D: After gadolinium ethoxybenzyl diethylenetriaminepentaacetic acid enhancement, the tumor was discernible in the arterial phase and was clearly detected as a hypo-intense lesion in the hepato-biliary phase on T1-weighted imaging. CE: Contrast enhanced.

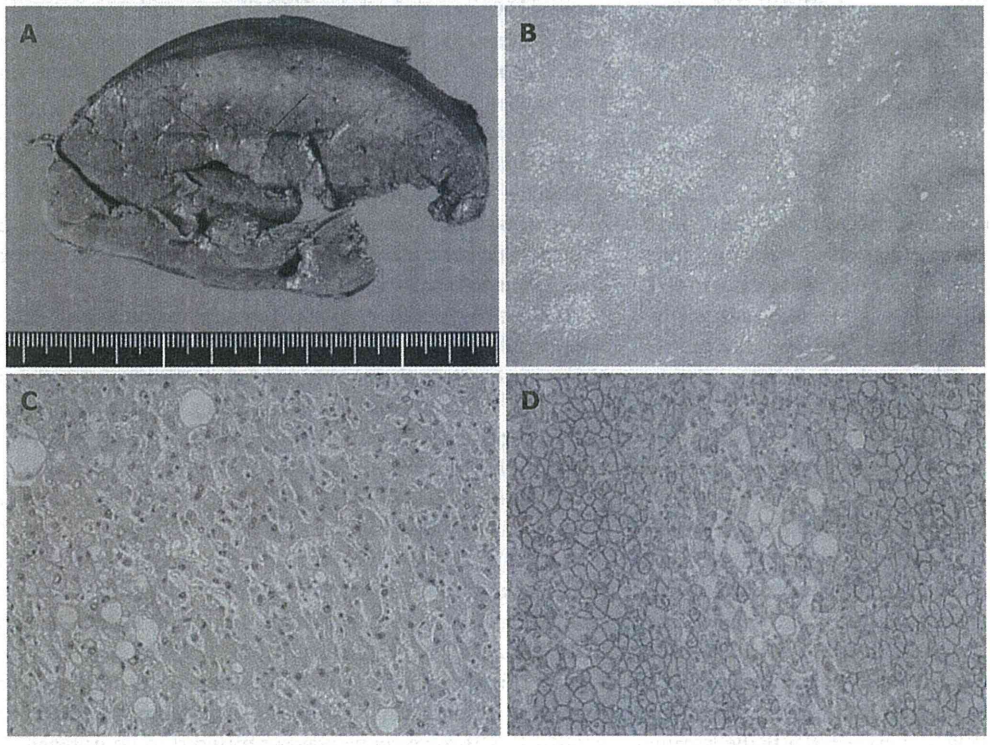

Figure 3 Pathological findings of the liver tumor. A: Cut surface of formalin-fixed liver specimen. The tumor was unencapsulated and its border was ill-defined (arrow); B, C: Microscopically, the tumor consisted of low-grade atypical hepatocytes, without cellular mitosis or changes in cellular density and structures. Fatty deposition in the tumor cells was ubiquitously remarkable. Neither biliary structures nor portal triads were present within the tumor (hematoxylin-eosin stain, original magnification ×40 in B and ×200 in C); D: β-catenin was immunohistochemically detected on the cytomembrane. Neither aberrant nuclear nor cytoplasmic accumulations were found (original magnification ×100).

tumor showed an ill-defined border and was unencapsulated (Figure 3A). The surrounding liver tissue seemed to be normal parenchyma.

Histologically, multiple colorectal polyps were adenomas with mild to moderate cellular atypia. A lateral spreading tumor was a tubular adenoma with severe atypia. The liver tumor consisted of low-grade atypical hepatocytes, without cellular mitosis or changes in cellular density and structures. Fatty deposition in the tumor cells was remarkable in some parts. No biliary structures or portal triads were present within the tumor (Figure 3B and C). There was no underlying hepatitis, fibrosis or cirrhosis in the adjacent liver parenchyma. These pathological findings were compatible for hepatic adenoma. To clarify the pathogen-

Table 1 Reported cases of primary hepatocellular adenoma associated with familial adenomatous polyposis

Case	Age gender	Location	No. of tumors	Size (cm)	Treatment	Oral contraception	Steroid use	Mutated codon in the *APC* gene	Disorder in the somatic gene of HCA
Bala *et al*[11]	2/F	Right lobe	Solitary	10	Resection	(-)	(-)	1451	Loss of wild-type allele of *APC*, mutation of *p53*
Nakao *et al*[13]	20/F	Left lobe	Multiple	5.5	Observation	(-)	(+)	ND	ND
Bläker *et al*[10]	27/F	ND	ND	ND	ND	ND	ND	1156	1516
Jeannot *et al*[12]	37/F	Right lobe	Solitary	7	Resection	(+)	(-)	1062	Mutation of *HNF1α*
Okamura *et al*[5]	27/M	Left lobe	Solitary	8.5	Resection	(-)	(-)	ND	ND
[1]Toiyama *et al*[14]	25/M	Left lobe	Solitary	5.5	Resection	(-)	(-)	ND	Mutation of *HNF1α*
This case	29/F	Right lobe	Solitary	3	Resection	(-)	(-)	499	(-)

[1]Case 6 is hepatocellular carcinoma within hepatocellular adenoma (HCA) in a familial adenomatous polyposis patient. *APC*: Adenomatous polyposis coli; ND: Not described; *HNF1α*: Hepatic nuclear factor 1 alpha; F: Female; M: Male.

esis of this patient, genetic alterations of the germ-line and somatic genes were examined[16]. Sequencing of the germline *APC* gene revealed a transition from ACG to ATG at codon 499 in exon 11. No loss of the *APC* gene in HCA cells was demonstrated by fluorescence in situ hybridization (data not shown). No additional somatic mutation of the *APC* gene was found in the HCA. Moreover, a mutation of the hepatocyte nuclear factor 1 alpha (*HNF1* gene, which is reported to be related to HCA[12]) was not detected.

The postoperative course was uneventful without any complications, and the patient was discharged twenty days after the operation. Follow-up CT scans revealed no signs of recurrence and other abdominal extracolic lesions 3 years after surgery.

DISCUSSION

HCA is usually found in healthy young women, especially those who use oral contraceptives for a long time. More than 750 HCA cases have been reported since the first report, showing a possible etiological association between HCA and contraceptives[17]. Glycogen-storage diseases, androgens, anabolic steroids, diabetes mellitus, some drugs and pregnancy have been reported as other causal factors for HCA[2-5]. However, the present patient did not have any known exogenous or endogenous pathogenic factors, except for FAP.

Patients with FAP can develop extracolonic lesions such as desmoid tumors, adenomas and carcinomas of the upper gastrointestinal tract[6]. An increased risk of hepatic tumors, mainly hepatoblastoma and hepatocellular carcinoma[18-20], has also been shown in FAP patients. Hepatoblastomas develop in young patients with FAP at least 100 times more frequently than in the general population[18]. Kurahashi *et al*[19] reported a biallelic mutation in the *APC* gene in hepatoblastoma developed in a FAP patient showing a germline mutation in *APC*. In fact, this patient's son had hepatoblastoma at the age of 18 mo but the precise genetic information of hepatoblastoma has not been obtained.

Reported cases of HCAs arising in FAP patients are extremely rare. According to our literature review, only seven cases, including our case, have been reported (Table 1)[5,10-14]. Five of these patients were female and two were male. Among them, one patient used oral contraceptives[12] and another had a medical history of androgenic steroid use for the treatment of anaplastic anemia[13]. HCA containing HCC in a male FAP patient was recently presented[14].

The germ-line mutation of the *APC* gene was examined in four cases, including our patient[10-12]. Bala *et al*[11] suggested that inherited mutations in the *APC* gene between codon 1444 and 1578 significantly increase the risk of developing extraintestinal tumors, including liver tumors. However, the other *APC* gene mutation occurred at different codons in 3 cases[10,12], including the present case (Table 1). Biallelic inactivation of the *APC* gene was described in two cases[10,11] (Table 1). In the first case, loss of the wild-type *APC* allele, which caused hemizygosity of the inherited mutation, was demonstrated[11]. A somatic 4-bp insertion was detected at codon 1516 in another case[10]. These findings suggest that the relationship between the *APC* gene anomaly and HCA is more complicated than initially expected.

Recently, genotype/phenotype classifications of HCA have drawn attention as a noticeable phenomenon from the aspects of pathogenesis and pathological tumorigenesis[21-23]. In their reports, HCAs are classified into four categories: (1) HCAs with mutations of the *HNF1* gene (H-HCA, 35%-40%); (2) HCAs with mutations of the *β-catenin* gene (β-HCA, 10%-15%); (3) inflammatory HCAs with mutation of the *IL6ST* gene (I-HCA, 40%-50%); and (4) HCAs without markers (unclassified HCA, less than 5%-10%). Our patient showed no symptoms or signs of an inflammatory syndrome. Additionally, the HCA in the present case morphologically lacked the typical characteristics of I-HCA, such as inflammatory infiltrates, sinusoidal dilatation and numerous thick arteries[21-23]. The *β-catenin* gene was supposed to be normal[24,25] because *β-catenin* was immunohistochemically detected only around the cytomembrane, without aberrant nuclear and cytoplasmic staining distributed in random and heterogeneous patterns (Figure 3D). Thus, the tumor is not β-HCA. In our case, histopathological characteristics of the liver tumor were closely compatible for H-HCA (Figure 3B and C) since H-HCAs are pathologically characterized by marked lipid deposition in tumor cells without

cytological abnormalities or inflammatory infiltrates[21-23]. However, no *HNF1* gene mutation was identified (data not shown). Although this tumor may be categorized as an unclassified HCA, further investigation of tumorigenesis is necessary[26].

In conclusion, we reported here a rare case of HCA arising in a female FAP patient. Because of its rarity, the pathogenesis of HCAs in patients with FAP remains undefined. More cases should be examined to establish the genetic alterations associated with benign hepatic tumorigenesis in FAP patients. Results may shed light on a breakthrough for hepatocellular carcinogenesis[25].

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Lipid peroxidation-induced DNA adducts in human gastric mucosa

Tomonari Matsuda[1], Hong Tao[2], Masanori Goto[2], Hidetaka Yamada[2], Masaya Suzuki[2], Yijia Wu[3], Nong Xiao[4], Qiong He[5], Wenwen Guo[6], Zhenming Cai[6], Nobuya Kurabe[2], Keiko Ishino[2], Yoshitaka Matsushima[7], Kazuya Shinmura[2], Hiroyuki Konno[8], Masato Maekawa[9], Yaping Wang[6] and Haruhiko Sugimura[2,*]

[1]Research Center for Environmental Quality Management, Kyoto University, Otsu, Shiga 520–0811, Japan, [2]Department of Tumor Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431–3192, Japan, [3]Department of Gastroenterology, Lujiang People Hospital, 32 Wenmingzhong Road, Lujiang, Anhui 231501, China, [4]Department of Internal Medicine, Lujiang People Hospital, 32 Wenmingzhong Road, Lujiang, Anhui 231501, China, [5]Department of Pathology, Lujiang People Hospital, 32 Wenmingzhong Road, Lujiang, Anhui 231501, China, [6]Jiangsu Key Laboratory of Molecular Medicine, Nanjing University School of Medicine, 22 Hankou Road, Nanjing 210093, China, [7]Department of Human Science, Chemistry Division, Hamamatsu University School of Medicine, Hamamatsu 431–3192, Japan, [8]Department of Surgery II, Hamamatsu University School of Medicine, Hamamatsu 431–3192, Japan and [9]Department of Laboratory Medicine, Hamamatsu University School of Medicine, Hamamatsu 431–3192, Japan

*To whom correspondence should be addressed. Tel: +81-53-435-2220; Fax: +81-53-435-2225;
Email: hsugimur@hama-med.ac.jp

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,*N*6-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 10^9 bases. Although the presence of *Helicobacter pylori* DNA in the mucosa was not related to these adducts level, 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 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: BedA, butanone-etheno-2′-deoxyadenosine; BedC, butanone-etheno-2′-deoxycytidine; BemedC, butanone-etheno-2′-deoxy-5-methylcytidine; edA, 1,N6-etheno-2′-deoxyadenosine; HedA, heptanone-etheno-2′-deoxyadenosine; HedC, 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

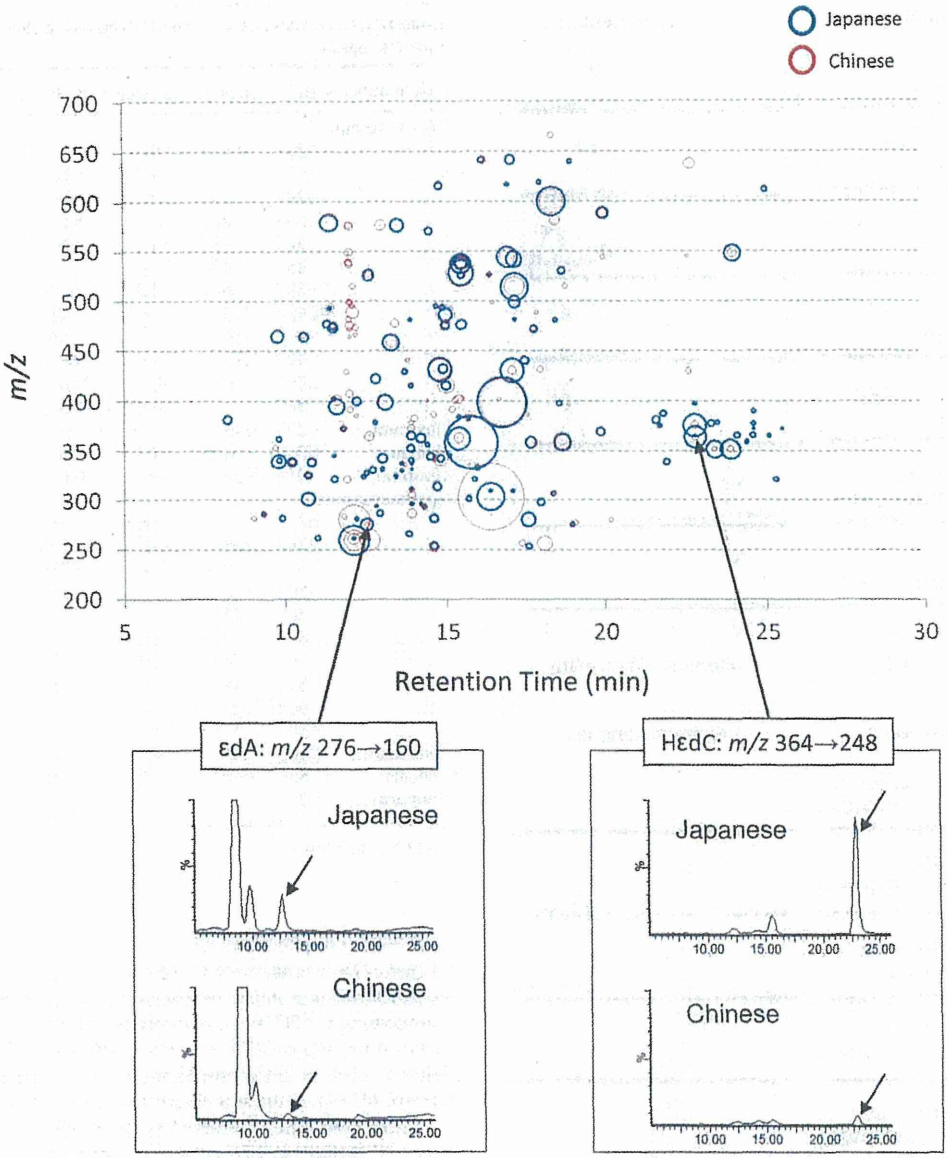

Fig. 1. Pooled-DNA adductome map of gastric mucosa samples from 10 Japanese and 10 Chinese patients. In this plot, the bubble size represents the relative peak area of MS/MS chromatogram. The liquid chromatography and double mass spectrometry peaks corresponding to the lipid peroxidation-induced DNA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

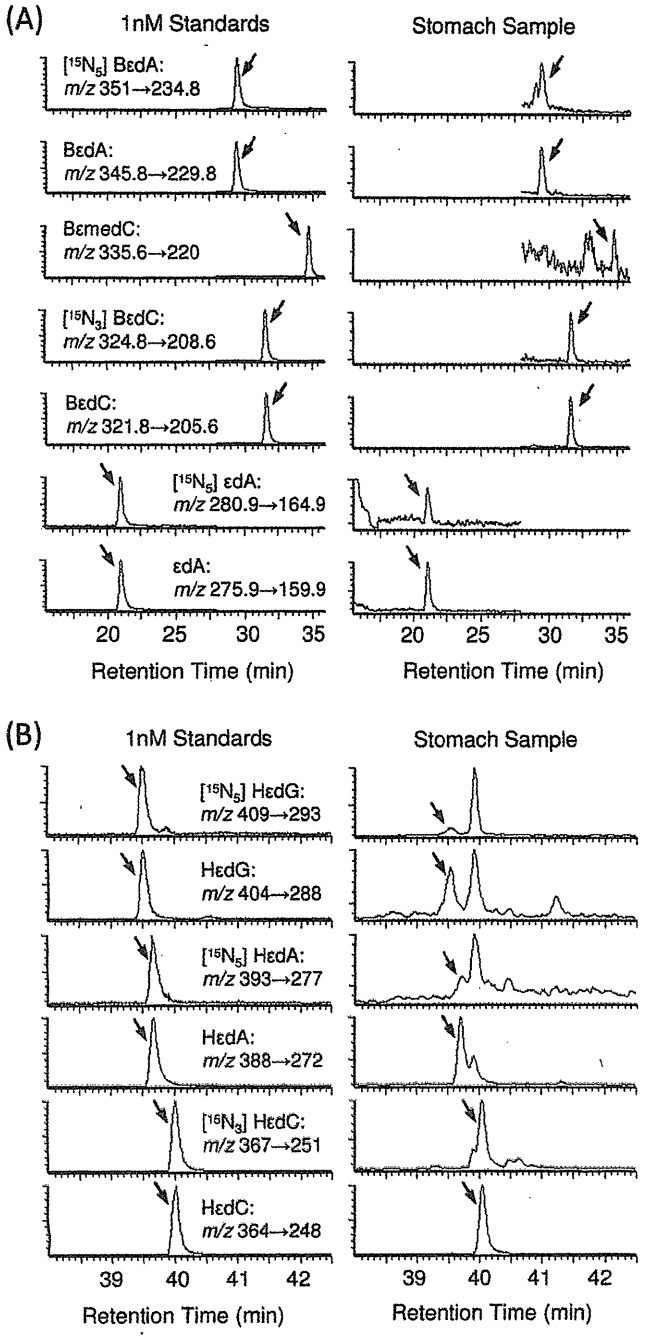

Fig. 2. Representative liquid chromatography and double mass spectrometry chromatogram of the lipid peroxidation-induced DNA adducts. Chromatogram of 1 nM standards (left panel) and a stomach sample (right panel) were shown. This sample is from the case number 21 of Table II. (**A**) Chromatogram of 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

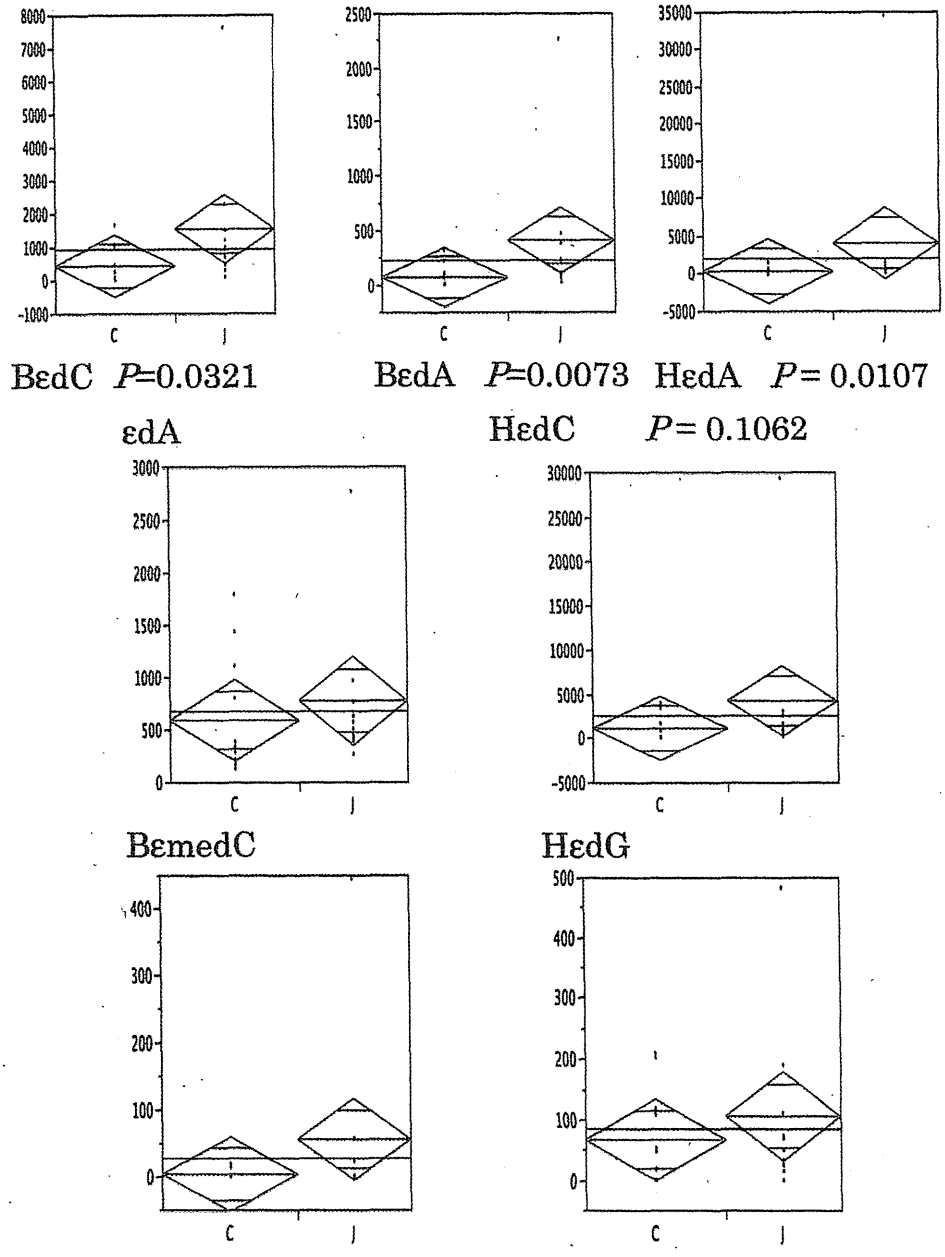

Fig. 3. Comparison of each adduct level between the Lujiang (C) and Hamamatsu (J) cases. The levels of BεdC, BεdA and HεdA were significantly higher for the Hamamatsu cases ($P < 0.05$ according to a Wilcoxon rank sum test). The horizontal lines in the diamonds indicate the upper quadrant, the mean and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