Jpn. J. Cancer Res. **90**, 1104–1108, October 1999

Detection of Methylation Damage in DNA of Gastric Cancer Tissues Using ^{32}P-Postlabelling Assay

Dae-Yong Kim,[1] Myung-Haing Cho,[2] Han-Kwang Yang,[3] Kari Hemminki,[4] Jin-Pok Kim,[3] Ja-June Jang[5] and Rajiv Kumar[4]

[1]Department of Veterinary Pathology, [2]Toxicology, College of Veterinary Medicine, [3]Department of Surgery and [5]Pathology, College of Medicine, Seoul National University, Suwon, 441-744, Korea and [4]Center for Nutrition and Toxicology, Karolinska Institute, 14157 Huddinge, Sweden

Gastric cancer is the most common cancer in Korea. The causes are still unknown but it has been speculated that gastric cancer is associated with consumption of foods rich in nitrates/nitrites or a high dietary intake of salt or pickled food. In the present study, we studied the level of alkylated DNA adducts formed in gastric cancer tissues in comparison with that in normal gastric mucosa. DNA was extracted from surgically removed gastric cancer tissues and patient-matched normal gastric mucosa. The level of N^7-methyldeoxyguanosine was measured by ^{32}P-postlabelling assay after high performance liquid chromatography (HPLC) enrichment. We found that the level of N^7-methyldeoxyguanosine of gastric cancerous tissues was significantly higher than that of normal gastric mucosa (P=0.01685).

Key words: Gastric cancer — DNA adduct — ^{32}P-Postlabelling — DNA damage

In spite of a marked decrease of the incidence of gastric cancer in many countries, gastric cancer still remains as one of the most common causes of cancer-related death in the world.[1, 2] In Korea, gastric cancer is the most frequently seen malignancy and the most important cause of cancer-related death.[3] One in four patients with a malignant tumor has been diagnosed as having gastric cancer. According to epidemiological and experimental animal studies, environmental factors and life-style, including dietary habits, have been blamed as the primary risk factor of gastric cancer.[4–6] Even though there is still some controversy, it has been proven that human gastric cancer is strongly associated with consumption of foods rich in nitrates/nitrites, along with high salt intake.[5, 6] In fact, fresh vegetables, fruit, and soybeans, which can prevent nitrosamine formation, are known to have a strong chemopreventive effects on human gastric carcinogenesis.[5, 6]

N-Nitroso compounds are well-known potential human carcinogens and can cause a variety of tumors in animals as well, by forming alkylated DNA adducts.[7] So far, more than 200 N-nitroso compounds have been found and humans are continuously exposed to many of these compounds through the diet, smoking, occupation and during cancer chemotherapy. Nitrosamine can also formed endogenously by nitric oxide released from inflammatory cells associated with *Helicobacter (H.) pylori* infection.[8]

Various molecular-biological techniques have been developed and are being used to detect and measure the degree of DNA damage caused by suspected carcinogens in target tissues.[9, 10] This so-called "molecular dosimetry" can predict the risk of cancer related to dietary habit, life style, or occupation, and is a valuable tool for studying the role of DNA adducts in cancer development. This study was performed to investigate the degree of alkylation damage to DNA in gastric cancer tissues of Korean patients, using combined high performance liquid chromatography (HPLC) and ^{32}P-postlabelling assay. N^7-Methylguanine was used as a marker to study the degree of DNA alkylation.

MATERIALS AND METHODS

Study subjects A total of 7 samples were collected during partial or total gastrectomy of gastric cancer patients. None of the patients had received any chemotherapy or radiotherapy prior to surgery. Immediately after removal, about 0.5 g of tumor tissues and normal gastric mucosa distant from the cancerous area was snap-frozen in liquid nitrogen and kept at −70°C until analysis. The rest of tissues were fixed in 10% neutral phosphate-buffered formalin, and embedded in paraffin, then sections were made for histopathological examination. This study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of Seoul National University Hospital.

DNA extraction and enzymatic hydrolysis Frozen cancer and normal mucosal samples were powdered in liquid nitrogen and digested overnight at 37°C with RNase A (150 μg/ml), RNase T1 (50 U/ml), 1% sodium dodecyl sulfate followed by proteinase K (300 μg/ml) for 12 h in 4

[1]To whom correspondence and reprints requests should be addressd.
E-mail: daeyong@plaza.snu.ac.kr

vol of digestion buffer (50 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0). DNAs were isolated by conventional phenol/chloroform/isoamyl alcohol (25:24:1) extraction and were precipitated with ice-cold ethanol and 0.1 *M* sodium acetate. The purity and concentration of DNAs were determined by measuring the UV absorbance at 260, 280, and 320 nm using a UV spectrophotometer (Jasco, Tokyo). Purified DNA samples (50–100 μg in 50 μl water) were enzymatically hydrolyzed to nucleoside-3′-monophosphates by incubating the DNA with reaction buffer (20 μl; 25 m*M* Tris-HCl, 25 m*M* $CaCl_2$, pH 8.8) containing 10 U of micrococcal nuclease for 30 min at 37°C. Further digestion was done with spleen phosphodiesterase at 37°C for another 90 min.

HPLC purification and enrichment of adducts Enzymatically hydrolyzed nucleoside-3′-monophosphates and standard adduct (N^7-methyldGp) were separated by HPLC (Kontron, Milano, Italy) with an ion-pair column (Beckman, Palo Alto, CA) as previously described.[11] Collected N^7-methyldGp and dGp fractions were concentrated by vacuum centrifugation (Uniequip, München, Germany) and kept at −70°C until postlabelling assay.

^{32}P-Postlabelling assay Adduct (dissolved in 5 μl of water) and internal standard (10 pmol dGp) were incubated for 1 h at 37°C with 10 U of T^4-PNK and 20 μCi of [γ^{32}]ATP (2 μl; 20 μCi) in 10 m*M* bicine, pH 8.6 containing 10 m*M* $MgCl_2$ and 1 m*M* spermidine followed by another 10 min treatment with nuclease P1 at 37°C.[12] Five microliter aliquots of the mixtures were applied to polyethylamine cellulose plates (20 cm × 20 cm, Selecto Scientific, Norcross, GA) together with UV-detectable amounts of internal standard. Two-dimensional thin-layer chromatography (TLC) was performed using ammonium acetate/isopropanol (90:10) as D1 and saturated ammonium sulfate/isopropanol/sodium acetate (80:2:18) as D2. After development, the TLC plates were exposed to Imaging plates (Fuji, Minami-Ashigara) and the radioactivity was counted using a radioanalytical image detection system (BAS 2500, Fuji, Tokyo). The molar ratio of radiolabelled N^7-methyl-2′-deoxyguanosine-5′-monophosphate (N^7-me-pdG) to pdG was calculated and extrapolated back to the starting amount of dGp as determined by UV during HPLC.

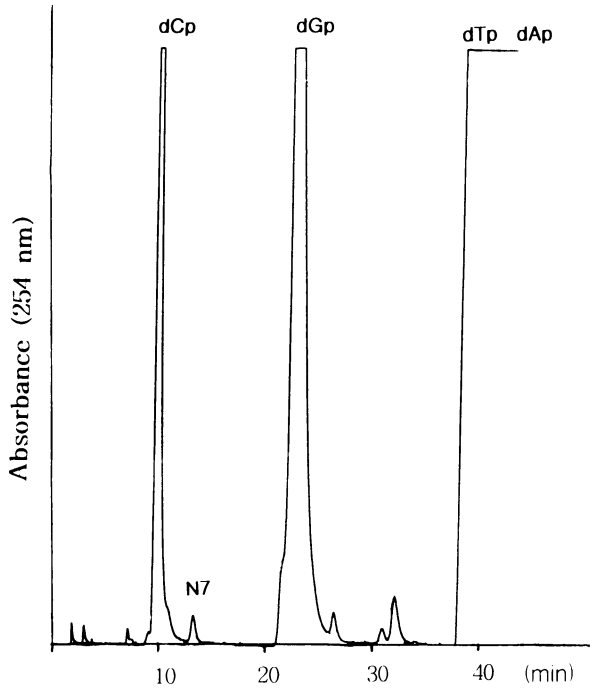

Fig. 1. HPLC elution profile of DNA extracted from human gastric cancer. N7, N^7-methyl-2′-deoxyguanosine-3′-monophosphate; dCp, 2′-deoxycytidine-3′-monophosphate; dGp, 2′-deoxyguanosine-3′-monophosphate; dTp, 2′-deoxythymidine-3′-monophosphate; dAp, 2′-deoxyadenosine-3′-monophosphate.

Table I. Relative Ratio of N^7-me-pdG to dGp in Cancerous and Patient-matched Noncancerous Tissues Taken from 7 Gastric Cancer Patients

Patient	Cancer tissue	Adjacent tissue
1	7.93×10^{-4}	4.25×10^{-4}
2	9.03×10^{-4}	4.43×10^{-4}
3	3.30×10^{-4}	1.10×10^{-4}
4	11.4×10^{-4}	4.43×10^{-4}
5	2.41×10^{-4}	1.12×10^{-6}
6	2.07×10^{-4}	8.07×10^{-5}
7	7.91×10^{-5}	5.67×10^{-5}

RESULTS

Histopathology All 7 cancer specimens used in this study for ^{32}P-postlabelling analysis were confirmed histologically as gastric adenocarcinoma. All of them were intestinal in type.

^{32}P-Postlabelling assay Fig. 1 shows the HPLC elution profile of mononucleoside-3′-phosphates obtained from gastric DNA. N^7-MethyldGp was eluted about 13.7 min after injection. Relative ratios of N^7-me-pdG to total amount of dGp in gastric tissues taken from 7 patients are shown in Table I. The mean values of the ratio of N^7-me-pdG to dGp were $5.28\times10^{-4}\pm1.55\times10^{-4}$ (mean±standard error) and $1.83\times10^{-4}\pm0.73\times10^{-4}$ for tissues taken from the cancerous area and normal mucosa, respectively (Fig. 2). The amounts of N^7-me-pdG adduct present in the tumor tissue were significantly higher than that present in the normal mucosa by paired *t*-test (P=0.01685).

Representative autoradiographs of the standard N^7-me-pdG and DNA extracted from cancer tissue following ^{32}P-postlabelling are shown in Fig. 3.

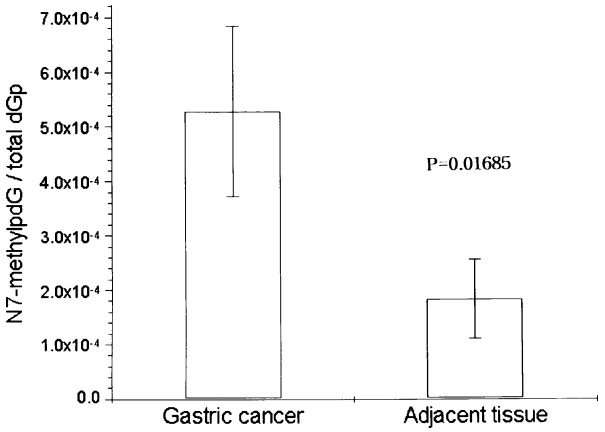

Fig. 2. Mean relative ratio of N^7-me-pdG to total dGp in 7 paired human gastric cancer and adjacent normal tissues. The total amount of dGp was determined by HPLC. The level of significance of the difference between tumoral and nontumoral adjacent tissues was tested by paired *t* test (P=0.01685).

DISCUSSION

Gastric cancer is the most common cause of cancer-related death in Korea.[3] Life style (dietary pattern or tobacco smoking), environmental or occupational exposure to various carcinogens can contribute to the development of gastric cancer.[5, 6, 8] Many lines of epidemiological evidence suggest that consumption of foods rich in nitrates / nitrites and salt or infection with *H. pylori* are associated with high risk of gastric cancer. In contrast to diffuse-type adenocarcinoma, of which the etiology is still unknown, the intestinal type of gastric carcinoma is thought to be linked with environmental factors prevalent early in life.[1] In fact, in Korea, the majority of gastric adenocarcinomas are intestinal in type and all 7 specimens used in this study were histologically diagnosed as intestinal type.

N-Nitroso compounds are known to cause point mutation by base substitution due to mispairing at loci where alkylated adducts are formed.[7] Certain N-nitroso compounds can also cause DNA strand breaks or sister chromatid exchange. Mutations of the H-*ras* oncogene were found in rat mammary tumors when the rats were exposed to N-methyl-N-nitrosourea (NMU).[13] Induction of brain

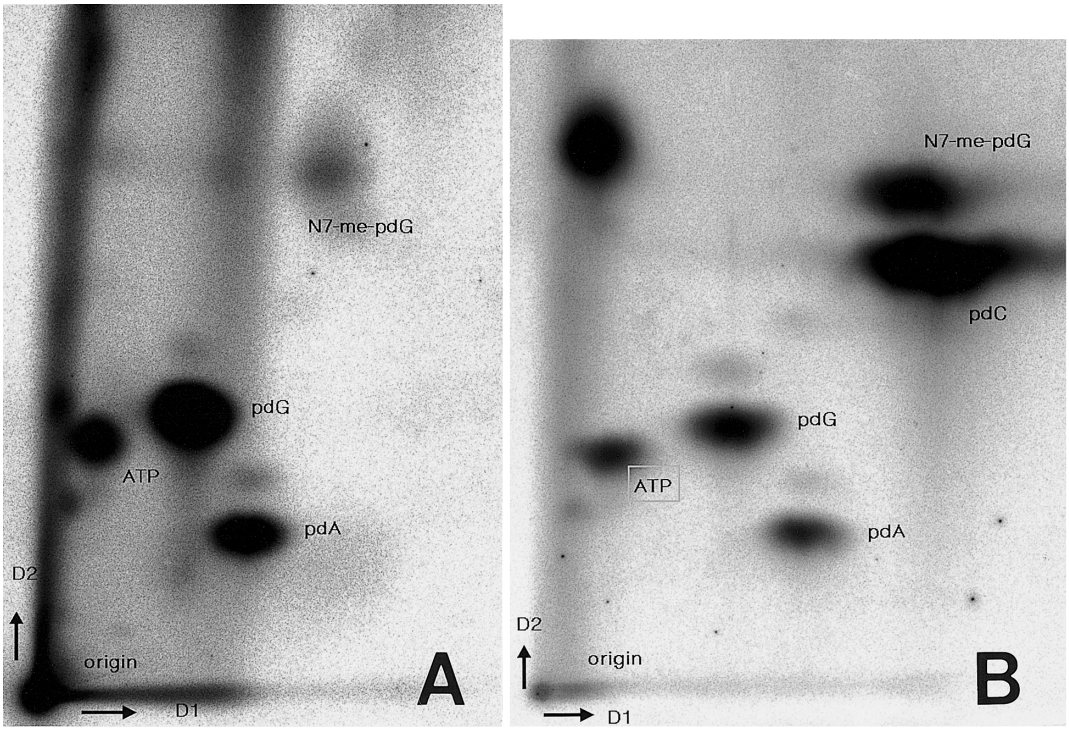

Fig. 3. Autoradiographs of N^7-methyl-2′-deoxyguanosine-5′-monophosphate. A, control standard; B, gastric cancer tissue. N7-me-pdG, N^7-methyl-2′-deoxyguanosine-5′-monophosphate; pdA, 2′-deoxyadenosine-5′-monophosphate; pdG, 2′-deoxyguanosine-5′-monophosphate; ATP, adenosine-5′-triphosphate.

tumors of rats by ethylnitrosourea has been correlated with the persistence of O^6-ethylguanine.[14] Indeed, the incidence of thymic lymphoma in mice transgenically transfected with human O^6-alkylguanine-DNA alkyltransferase was significantly lower than that of a nontransfected control group when they were exposed to NMU.[15] It is generally accepted that the levels of DNA adducts in target tissues during chronic exposure seem to be carcinogen dose-related and could be predictive of human cancer risk.[16, 17]

In Lin Xian, a district of northern China, where the incidence of gastric and esophageal cancer is high, the level of O^6-methylguanosine present in gastric and esophageal cancer tissues was 3 times and 1.5 times higher than those of control groups, respectively, when measured by radioimmunoassay.[18] Furthermore, the concentrations of N-nitrosamine and nitrate present in the urine of people living in Lin Xian county were higher than in people living in Fanxian county.[19] A similar observation has also been reported from the United Kingdom.[20] According to Huh *et al.*, the mean hepatic O^4-ethylthymine level was about 4 to 5 times higher in patients with malignant liver cancer than in patients with non-malignant diseases.[21] In our present study, the mean N^7-methylguanine level of gastric cancer tissues was about 2.5 times higher than those of normal gastric mucosa. Our observation is consistent with previous findings and supports the hypothesis that DNA damage owing to alkylation is involved in human gastric carcinogenesis.

Considering the strong alkylating potential of N-nitroso compounds, whether they are formed endogenously or exogenously, those compounds might play some role in the development of gastric cancer by causing mutations of various oncogenes or tumor suppressor genes known to be involved in gastric cancer following adduct formation.

Due to the limited sample size, the direct correlation between life style, nutrition or effects of genetic polymorphism of metabolic enzymes and adduct levels of the patients could not be analyzed.[22] However, the results of our study suggest that further investigation using larger and well-characterized cohorts of patients would be warranted to better delineate the role and significance of alkylation damage to DNA in human gastric carcinogenesis.

ACKNOWLEDGMENTS

The authors thank Dr. Dae-Young Kim for technical assistance. This study was supported by a Grant-in-Aid from the Korea Tobacco and Ginseng Corporation.

(Received May 18, 1999/Revised July 27, 1999/Accepted July 30, 1999)

REFERENCES

1) Fuchs, C. S. and Mayer, R. J. Gastric carcinoma. *N. Engl. J. Med.*, **333**, 32–41 (1995).
2) Cady, B., Ross, R. and Silverman, M. Gastric adenocarcinoma. A disease in transition. *Arch. Surg.*, **124**, 303–308 (1989).
3) Ahn, Y.-O., Park, B.-J., Yoo, K.-Y., Kim, N.-K., Heo, D.-S., Lee, J.-K., Ahn, H.-S., Kang, D.-H., Kim H., Lee M.-S. and Park, T.-S. Incidence estimation of stomach cancer among Koreans. *J. Korean Med. Sci.*, **6**, 7–14 (1991).
4) Tatematsu, M., Takahashi, M., Hanaouchi, M. and Shirai, T. Effects in rats of sodium chloride on experimental gastric cancers induced by N-methyl-N-nitro-N-nitroguanidine or 4-nitroquinoline-1-oxide. *J. Natl. Cancer Inst.*, **55**, 101–106 (1985).
5) Ramon, J. M., Serra, L., Cerdo, C. and Oromi, J. Dietary factors and gastric cancer risk. *Cancer*, **71**, 1731–1735 (1993).
6) Risch, H. A., Jain, M., Choi, N. W., Fodor, J. G., Pfeiffer, C. J., Howe, G. R., Harrison, L. W., Craib, K. J. and Miller, A. B. Dietary factor and the incidence of cancer of the stomach. *Am. J. Epidemiol.*, **122**, 947–959 (1985).
7) Hemminki, K. DNA adducts, mutations and cancer. *Carcinogenesis*, **14**, 2007–2012 (1993).
8) Ohshima, H. and Bartsch, H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res.*, **305**, 253–264 (1994).
9) Cadet, J. and Weinfeld, M. Detecting DNA damage. *Anal. Chem.*, **65**, 675–682 (1993).
10) Shields, P. G. and Harris, C. C. Molecular epidemiology and the genetics of environmental cancer. *JAMA*, **266**, 681–687 (1991).
11) Shields, P. G., Povey, A. C., Wilson, V. L., Weston, A. and Harris, C. C. Combined high-performance liquid chromatography/^{32}P-postlabelling assay of N^7-methyldeoxyguanosine. *Cancer Res.*, **50**, 6580–6584 (1990).
12) Haque, K., Cooper, D. P. and Povey, A. C. Optimization of ^{32}P-postlabelling assay for the quantitation of O^6-methyl and N^7-methydeoxyguanosine-3′-monophosphate in human DNA. *Carcinogenesis*, **15**, 2485–2490 (1994).
13) Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zance, D. and Barbacid, M. Direct mutagenesis of H-*ras*-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. *Nature*, **315**, 382–385 (1985).
14) Goth, R. and Rajewsky, M. F. Persistence of O^6-ethylguanine in rat brain DNA: correlation with nervous system-specific carcinogenesis by ethylnitrosourea. *Proc. Natl. Acad. Sci. USA*, **71**, 639–643 (1974).
15) Dumenco, L. L., Allay, E., Norton, K. and Gerson, S. L. The prevention of thymic lymphomas in transgenic mice by human O^6-alkylguanine-DNA alkyltransferase. *Science*, **259**, 219–222 (1993).
16) Poirier, M. C. and Beland, F. A. DNA adduct measurement and tumor incidence during chronic carcinogen exposure in animal models: implication for DNA adduct-based human

cancer risk assessment. *Chem. Res. Toxicol.*, **5**, 749–755 (1992).
17) Ross, J. A., Nelson, G. B., Wilson, K. H., Rabinowitz, J. R., Galati, A., Stoner, G. D., Nesnow, S. and Mass, M. J. Adenomas induced by polycyclic aromatic hydrocarbons in strain A/J mouse lung correlate with time-integrated DNA adduct levels. *Cancer Res.*, **55**, 1039–1044 (1995).
18) Umbenhauer, D., Wild, C. P., Montesano, R., Saffhill, R., Boyle, J. M., Huh, N. H., Kirstein, U., Thomale, J., Rajewsky, M. F. and Lu, S. H. O^6-Methyldeoxyguanosine in oesophageal DNA among individuals at high risk of oesophageal cancer. *Int. J. Cancer*, **36**, 661–665 (1985).
19) Lu, S. H., Ohshima, H., Fu, H. M., Tian, Y., Li, F. M., Blettner, M., Wahrendorf, J. and Bartsch, H. Urinary excretion of N-nitrosamino acids and nitrate by inhabitants of high- and low-risk areas for esophageal cancer in Northern China : endogenous formation of nitrosoproline and its inhibition by vitamin C. *Cancer Res.*, **46**, 1485–1491 (1986).
20) Hall, C. N., Badawi, A. F., O'Connor, P. J. and Saffhill, R. The detection of alkylation damage in the DNA of human gastrointestinal tissues. *Br. J. Cancer*, **64**, 59–63 (1991).
21) Huh, N. H., Satoh, M. S., Shiga, J., Rajewsky, M. F. and Kuroki, T. Immunoanalytical detection of O^4-ethylthymine in liver DNA of individuals with or without malignant tumors. *Cancer Res.*, **49**, 93–97 (1989).
22) Wang, Y., Ichiba, M., Iyadomi, M., Zhang, J. and Tomokuni, K. Effects of genetic polymorphism of metabolic enzymes, nutrition, and lifestyle factors on DNA adduct formation in lymphocytes. *Ind. Health*, **36**, 337–346 (1998).