

SHORT COMMUNICATION

Safrole-like DNA adducts in oral tissue from oral cancer patients with a betel quid chewing history

Chiu-Lan Chen¹, Chin-Wen Chi^{1,3}, Kuo-Wei Chang² and Tsung-Yun Liu^{1,3,4}

¹Institute of Pharmacology and ²Institute of Oral Biology, National Yang-Ming University and ³Department of Medical Research and Education, Veterans General Hospital–Taipei, Taipei, Taiwan, Republic of China

⁴To whom correspondence should be addressed at: Department of Medical Research and Education, Veterans General Hospital–Taipei, Taipei, Taiwan 11217, Republic of China
Email: tyliu@vghtpe.gov.tw

Betel quid (BQ) chewing has been associated with an increased risk of oral squamous cell carcinoma (OSCC) and oral submucous fibrosis (OSF). *Piper betle* inflorescence, which contains 15 mg/g safrole, is a unique ingredient of BQ in Taiwan. Chewing such prepared BQ may contribute to safrole exposure in human beings (420 µM safrole in saliva). Safrole is a known rodent hepatocarcinogen, yet its carcinogenicity in human beings is largely undetermined. In this study, using a ³²P-post-labeling method, we have found a high frequency of safrole-like DNA adducts in BQ-associated OSCC (77%, 23/30) and non-cancerous matched tissue (NCMT) (97%, 29/30). This was in contrast to the absence (< 1/10⁹ nucleotides) of such adducts in all of non-BQ-associated OSCC and their paired NCMT (*P* < 0.001). Six of seven OSF also exhibited the same safrole-like DNA adduct. The DNA adduct levels in OSF and NCMT were significantly higher than in OSCC (*P* < 0.05). Using co-chromatography and rechromatography techniques, we further demonstrated that these adducts were identical to synthetic safrole–dGMP adducts as well as DNA adducts from 1'-hydroxysafrole-treated HepG2 cells. These results suggest that safrole forms stable safrole–DNA adducts in human oral tissue following BQ chewing, which may contribute to oral carcinogenesis.

Oral squamous cell carcinoma (OSCC) is one of the most common malignant neoplasms in Asian countries. It is the fifth leading cause of male cancer mortality in Taiwan (1). The International Agency for Research on Cancer concluded that OSCC is associated with betel quid (BQ) chewing together with tobacco or cigarette smoking (2). Oral submucous fibrosis (OSF) is also a frequent precancerous condition in BQ chewers (3). In Taiwan, tobacco is not included in the preparation used for BQ chewing. Nevertheless, epidemiological studies showed that BQ chewing is still the main cause of OSCC and OSF in Taiwan (4,5). The most popular way to chew BQ in Taiwan is a combination of the areca nut, *Piper betle* inflorescence (sometimes substituted by betel leaf) and lime paste. *Piper*

Abbreviations: BQ, betel quid; dAMP, 2'-deoxyadenosine 3'-monophosphate; dGMP, 2'-deoxyguanosine 3'-monophosphate; NCMT, non-cancerous matched tissue; OSCC, oral squamous cell carcinoma; OSF, oral submucous fibrosis; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

betle inflorescence contains a high concentration (15 mg/g) of safrole (6). Consequently, chewing BQ containing *Piper betle* inflorescence may contribute to safrole exposure (420 µM in saliva during chewing) (7).

Safrole is classified as a weak hepatocarcinogen in mice and rats (8). The carcinogenicity of safrole is mediated through 1'-hydroxysafrole formation, followed by sulfonation to an unstable sulfuric acid ester that reacts to form stable safrole–DNA adducts (9). 1'-Hydroxysafrole, the proximate carcinogen of safrole, was detected in the liver, urine and bile of animals treated with safrole (8). However, 1'-hydroxysafrole was not detected in human subjects who received 1.66 mg [¹⁴C]safrole (10). The reason for this discrepancy remains unknown. It is plausible that the administered dose or the assay sensitivity is insufficient for the detection of this metabolite. Using the ³²P-post-labeling technique, the most sensitive method for detection of DNA adducts (11), stable safrole–DNA adducts can be detected in both rodent liver and other tissues treated with safrole (12–14). However, the effect of safrole in human beings has not been documented. In this study, using the ³²P-post-labeling technique, we determined the presence of safrole-like DNA adducts in oral tissues of BQ users.

Histologically confirmed OSCC (*n* = 36) and OSF (*n* = 7) tissues were obtained from the Department of Dentistry, according to a protocol approved by the committee for the conduct of human research at the Veterans General Hospital–Taipei. Specimens were kept at –70°C immediately after excision until DNA extraction. History of BQ chewing, cigarette smoking, alcohol consumption and other clinical parameters were carefully recorded. Normal gingival tissues (*n* = 14) were obtained from healthy volunteers undergoing tooth extraction. DNA was extracted by a conventional phenol/chloroform procedure (15).

1'-Hydroxysafrole was synthesized from vinyl bromide and piperonal (16). 1'-Hydroxysafrole was then reacted with 2'-deoxyguanosine 3'-monophosphate (dGMP) or 2'-deoxyadenosine 3'-monophosphate (dAMP) in the presence of sulfotransferase and 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The incubation mixture contained 3 mM MgCl₂, 0.1 mM PAPS, 5 mM dGMP or dAMP, hepatic cytosol from ICR mice (2 mg protein) and 4 mM 1'-hydroxysafrole in a final volume of 2 ml of 50 mM Tris–HCl buffer (pH 7.4). After incubation at 37°C for 3 h, 5-fold cold acetone was added and the mixture was centrifuged at 10 000 g at 4°C for 20 min. The supernatant was analyzed by the ³²P-post-labeling assay. HepG2 cells were first cultured in DMEM medium containing 10% fetal bovine serum, then changed to serum-free medium and incubated with 400 µM 1'-hydroxysafrole for 24 h prior to adduct analysis.

Four micrograms of coded DNA samples were assayed by the nuclease P1-enhanced ³²P-post-labeling procedure as described by Reddy and Randerath (13). The [³²P]ATP (6000 Ci/mmol; NEN Life Science, Boston, MA)-labeled safrole–DNA adducts were resolved on polyethyleneimine–

cellulose TLC plates (Machery-Nagel, Düren, Germany) with two different buffer systems. Development condition A consisted of 2.3 M sodium phosphate, pH 6.0 (D1) and 1.8 M lithium formate, 4.25 M urea, pH 3.5 (D2) (bottom to top); 0.36 M lithium chloride, 0.22 M Tris-HCl, 3.8 M urea, pH 8.0 (D3) and 1.7 M sodium phosphate, pH 6.0 (D4) (left to right). Safrole-DNA adducts were detected by autoradiography and quantified by scintillation counting. Adduct levels are expressed as relative adduct labeling (RAL) $\times 10^8$ values (11). The sensitivity of this assay was limited to 1 adduct/ 10^9 nucleotides. To compare the adducts detected in OSCC with safrole-DNA adducts in 1'-hydroxysafrole-treated HepG2 cells, the two DNA samples were mixed before enzymatic digestion and, using the above-mentioned condition A, the resulting chromatogram was compared with maps derived from individual DNA samples. These adducts were also compared by a different development condition designated condition B: 1.7 M sodium phosphate, pH 6.0 (D1); 1.9 M lithium formate, 3.8 M urea, pH 3.5 (D2); 0.36 M sodium phosphate, 0.23 M Tris-HCl, pH 8.0 (D3); 1.7 M sodium phosphate, pH 6.0 (D4) (17). To further characterize these adducts, excised adducts from individual chromatograms were extracted with 4 N ammonium hydroxide:isopropanol (1:1 v/v) and rechromatographed for further separation (18,19).

Thirty-six patients with OSCC were enrolled in this study. The sex difference (34 males, two females) closely resembles the distribution of BQ chewers (21:1) in the Taiwanese population (20). The most common primary site of OSCC was the buccal mucosa (14 cases) followed by the tongue (seven cases) and gingiva (five cases). The majority of BQ chewers (30 cases) were also cigarette smokers (27 cases). Seven male patients with OSF were also included in this study, all of whom were BQ chewers and cigarette smokers.

We detected two major and two minor DNA adducts on autoradiograms from 1'-hydroxysafrole-treated HepG2 cells under condition A (Figure 1A). The levels of spot 1 and spot 2 were 105.0 and 22.3 adducts/ 10^8 nucleotides, respectively. This map was qualitatively similar to DNA adducts found in safrole-treated mice as described by Reddy and Randerath (13). The two major safrole-DNA adducts have been identified as *N*²-(*trans*-isofuroyl)-2'-deoxyguanosine (spot 1) and *N*²-(safrolyl)-2'-deoxyguanosine (spot 2) (21). The nature of the two minor safrole-DNA adducts (spots 3 and 4) remains unclear. In this study, one major spot (spot 1) in the fingerprints of OSCC and non-cancerous matched tissue (NCMT) (Figure 1B and C) as well as in OSF samples was detected. In addition, one minor spot (spot 2) was occasionally noticed (Figures 1C and 2B and E). The chromatographic location of this major DNA adduct is qualitatively similar to spot 1 as shown by others (12-14). On the other hand, no adduct was detectable on the autoradiogram of the control gingival sample (Figure 1D). The location of the major DNA adducts in oral tissues was qualitatively similar to that of the safrole-DNA adduct (spot 1) from 1'-hydroxysafrole-treated HepG2 cells (Figure 1A). In this development system, interference by benzo[*a*]pyrene and other macromolecular DNA adducts which result from cigarette smoking was unlikely since these adducts are much more hydrophobic than safrole adducts (data not shown).

In order to identify the nature of the DNA adducts, DNA from OSCC was mixed with 1'-hydroxysafrole-treated HepG2 DNA, then digested and developed in TLC plates. Under condition A, the major and minor spots co-migrated with safrole-DNA adducts 1 and 2 (spots 1 and 2). Co-migration

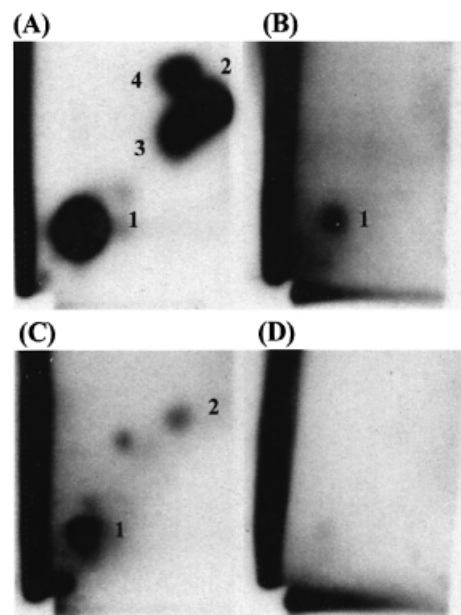


Fig. 1. Autoradiograms of polyethyleneimine-cellulose TLC maps of ³²P-labeled digests of DNA. (A) DNA from HepG2 cells treated with 400 μ M 1'-hydroxysafrole for 24 h; (B) DNA from an OSCC (patient no. 47); (C) DNA from NCMT (no. 48); (D) DNA from normal gingival tissue. Adducts were visualized by autoradiography employing Kodak Biomax MR imaging film with an intensifying screen for 24 h at -70°C , except for (A), which was exposed for 6 h.

of such adducts with safrole-DNA adducts 1 and 2 was demonstrable under development condition B (data not shown). To characterize these adducts further, we reacted dGMP and dAMP with 1'-hydroxysafrole and separated the products in a TLC plate using development condition B (Figure 2A and D). As shown in Figure 2C and F, the adduct product co-migrated with adduct 1 obtained from safrole-dGMP, but not with dAMP derivatives (spot 5). The nature of spots 1 and 2 in OSCC were further demonstrated to be identical to the 1'-hydroxysafrole-treated DNA adducts by two different solvent systems in 1-dimensional TLC (Figure 3). The major (spot 1) and minor (spot 2) adducts were confirmed as chromatographically indistinguishable from safrole-dGMP adducts using two distinct development conditions, co-chromatography and rechromatography.

The safrole-like DNA adduct was present in 77% (23/30) of OSCC, 97% (29/30) of NCMT from OSCC patients and 86% (6/7) of OSF patients with BQ use. In contrast, this safrole-like DNA adduct was not detectable in gingival tissues from 14 persons without BQ chewing. The presence of this adduct in oral samples is highly correlated with BQ chewing (Fisher's exact test, $P < 0.001$). The safrole-like DNA adduct levels in OSCC, NCMT and OSF were 4.0 ± 0.9 (range 0-19.4), 9.7 ± 2.7 (range 0-65.3) and 7.8 ± 1.5 (range 0-11.7) per 10^8 nucleotides, respectively (Table I). The DNA adduct levels in OSF and NCMT were significantly higher than that in OSCC (Mann-Whitney rank sum test, $P < 0.05$).

The formation of covalent adducts of DNA is generally regarded as a critical event in the initiation of chemical carcinogenesis. Consequently, measurement of DNA adducts may be a powerful tool to demonstrate a possible association between carcinogen exposure and cancer risk. The ³²P-post-labeling assay combined with the nuclease P1 enhancement technique provides a highly sensitive method for detecting

Table I. The effect of BQ chewing on the presence and level of the safrole-like DNA adduct

Safrole-like DNA adduct	BQ chewing ^a					
	Yes			No		
	OSCC (n = 30)	NCMT (n = 30)	OSF (n = 7)	OSCC (n = 6)	NCMT (n = 6)	Normal gingiva (n = 14)
Present	23	29	6	0	0	0
Absent	7	1	1	6	6	14
Levels ^b						
Mean ± SE	4.0 ± 0.9	9.7 ± 2.7	7.8 ± 1.5			
Median ^c	2.2	5.0	9.1			
Range	0 ^d –19.4	0–65.3	0–11.7			

^a $P < 0.001$ by Fisher's exact test, for OSCC and NCMT of BQ chewers versus non-BQ chewers.

^bAdduct levels are expressed as adducts/ 10^8 nucleotides.

^c $P < 0.05$ by Mann–Whitney test, for OSCC versus NCMT and OSCC versus OSF.

^dThe sensitivity of this assay is $1/10^9$ nucleotides and adduct levels below the limit of detection are represented by 0.

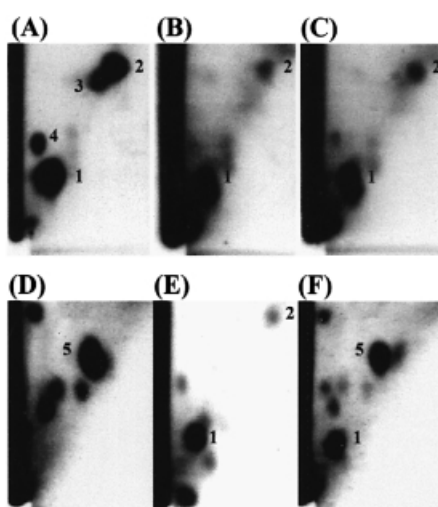


Fig. 2. Co-chromatography of ^{32}P -labeled safrole–DNA adducts. (A) dGMP reacted with 1'-hydroxysafrole; (B) DNA from an OSCC (no. 48); (C) A (1-fold) mixed with B (3-fold); (D) dAMP reacted with 1'-hydroxysafrole; (E) DNA from NCMT (no. 48); (F) D (1-fold) mixed with E (3-fold). Chromatographs were developed under condition B. Autoradiographs were obtained after 24 h at -70°C , except for (A), which was exposed for 10 h.

adducts in human tissue (11). Using this analysis, we demonstrated the presence of a safrole-like DNA adduct in oral DNA samples from OSCC and OSF patients and the presence of such an adduct is highly correlated with BQ chewing.

By giving safrole to mice, Randerath *et al.* reported that the loss of safrole adducts from hepatic DNA was biphasic: a rapid loss during the first week followed by a much slower decline for up to 20 weeks. The early rapid adduct loss was probably related to repair processes, while the latter may be due to cell turnover in carcinogen-exposed tissues rather than DNA repair (12). In this study, we detected one major and/or one minor adduct in oral tissues instead of four spots as seen in 1'-hydroxysafrole-treated HepG2 cells and mice hepatic tissues. There may be a number of reasons for this difference. First, adduct 1 forms more easily than other adducts. Second, adduct 1 is resistant to repair processes. Last, safrole requires a two-step metabolic activation to form DNA adducts and the metabolic capacity of oral tissue is lower than that of liver tissue. Consequently, the major adduct levels are lower and the minor adducts are not detectable in oral tissues.

Adduct levels in a cell are dependent on pharmacodynamic

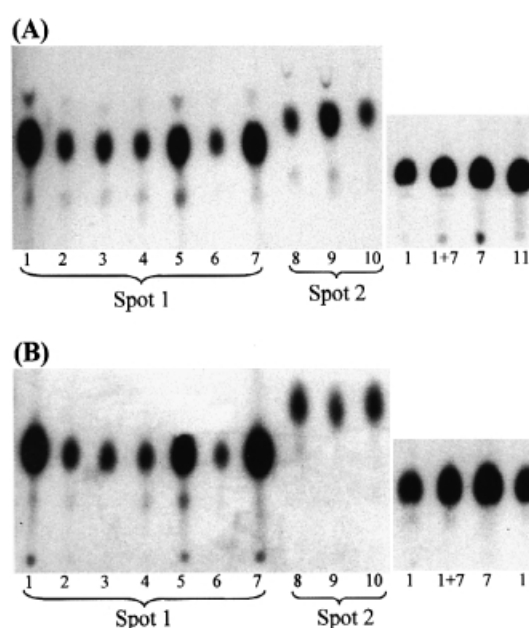


Fig. 3. Rechromatography analysis of ^{32}P -labeled safrole–DNA adducts. Solvents used for rechromatography were (A) 4 N ammonium hydroxide:isopropanol (1:1 v/v); (B) 0.65 M sodium chloride, 0.25 M boric acid, 0.005 M EDTA, 4 M urea, pH 8.0. OSCC tissue DNA (400 μg) was digested, nuclease P1 enriched and concentrated, then one fiftieth was labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 2-dimensional chromatography. Spot 1 was eluted from 2-dimensional TLC plates of DNA samples from six different OSCC tissues (lanes 1–6) and from 1'-hydroxysafrole-treated HepG2 cells (4 μg) (lane 7). Spot 2 was similarly excised and eluted from HepG2 cells (lane 8) and OSCC tissues (lanes 9 and 10). Lane 11 was sample 1 and 1'-hydroxysafrole-treated HepG2 DNA co-migrated in 2-dimensional TLC and then eluted for rechromatography. Lanes 1 and 9 and 5 and 10 are from the same individual. Adducts were visualized by autoradiography employing Kodak Biomax MR imaging film with an intensifying screen for 12 h at -70°C .

and pharmacokinetic factors, adduct stability and the rates of cell turnover and DNA repair. Substantial amounts of safrole-like DNA adducts were detected in most of the BQ-associated subjects and the levels of safrole-like DNA adducts were significantly higher in NCMT than in OSCC. Observations of a higher carcinogen–DNA adduct level in NCMT rather than tumors has been reported in lung, colorectal and breast cancer (18,22,23). A tentative explanation for this phenomenon is that the level of DNA adducts may have been diluted during

rapid DNA replication and tumor cell turnover. Another possibility is that the OSCC is less capable of activating the carcinogen; such a phenomenon has been observed in animal models (24).

Although the generation of safrole–DNA adducts in rodents is well defined (12–14), the role of these adducts in cancer development is not clear since there is no *in vivo* evidence highlighting the susceptibility to mutation of safrole–DNA adducts. Recently, Daimon *et al.* demonstrated that the genotoxic effects of safrole might result from covalent DNA modification in rat liver (14). However, the role of safrole–DNA adducts in non-target tissues and oral mucosa is not known at present. DNA adduction is known to be associated with the activation of proto-oncogenes. Activation of the *c-Ha-ras* proto-oncogene by point mutations in codon 61 has been found in hepatocellular carcinomas of safrole-treated B6C3F₁ mice (25), implicating the involvement of safrole-related DNA adducts in mutagenesis. The involvement of safrole–DNA adducts during oral carcinogenesis needs further study.

In conclusion, using the ³²P-post-labeling technique, we demonstrated the presence of safrole-like DNA adducts in oral tissues associated with BQ exposure. The results suggest that stable safrole–DNA adducts derived from *Piper betle* inflorescence, an ingredient of Taiwanese BQ, may be associated with oral carcinogenesis.

Acknowledgements

This study was supported in part by grants from the National Science Council (NSC 87-2314-B-075-029-M14) and Department of Health (DOH 88-HR-802), Republic of China.

References

- Anonymous (1998) *Cancer Registry Annual Report in Taiwan Area: 1995*. Department of Health, Executive Yuan, ROC.
- IARC (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*. Vol. 37. *Betel-Quid and Areca-Nut Chewing*. IARC, Lyon, pp. 141–202.
- Murti,P.R., Bhonsie,R.B., Gupta,P.C., Daftary,D.K., Pindorg,J.J. and Mehta,F.S. (1995) Etiology of oral submucous fibrosis with special reference to the role of areca nut chewing. *J. Oral Pathol. Med.*, **24**, 145–152.
- Ko,Y.C., Huang,Y.L., Lee,C.H., Chen,M.J., Lin,L.M. and Tsai,C.C. (1995) Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J. Oral Pathol. Med.*, **24**, 450–453.
- Shiau,Y.Y. and Kwan,H.W. (1979) Submucous fibrosis in Taiwan. *Oral Surg.*, **47**, 453–457.
- Hwang,L.S., Wang,C.K., Shen,M.J. and Kao,L.S. (1992) Phenolic compounds of *Piper betle* flower as flavoring and neuronal activity modulating agents. In Ho,C.T., Lee,C.Y. and Huang,M.T. (eds) *Phenolic Compounds in Food and Their Effects on Health I: Analysis, Occurrence and Chemistry*. American Chemical Society, Washington, DC, pp. 200–213.
- Wang,C.K. and Hwang,L.S. (1993) Phenolic compounds of betel quid chewing juice. *Food Sci.*, **20**, 458–471.
- Ioannides,C., Delaforge,M. and Parke,D.V. (1981) Safrole: its metabolism, carcinogenicity and interactions with cytochrome P-450. *Food Cosmet. Toxicol.*, **19**, 657–666.
- Miller,J.A. and Miller,E.C. (1983) The metabolic activation and nucleic acid adducts of naturally-occurring carcinogens: recent results with ethyl carbamate and the spice flavors safrole and estragole. *Br. J. Cancer*, **48**, 1–15.
- Benedetti,M.S., Malnoe,A. and Broillet,A.L. (1977) Absorption, metabolism and excretion of safrole in the rat and man. *Toxicology*, **7**, 69–83.
- Reddy,M.V. and Randerath,K. (1986) Nuclease P1-mediated enhancement of sensitivity of ³²P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543–1551.
- Randerath,K., Haglund,R.E., Phillips,D.H. and Reddy,M.V. (1984) ³²P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis*, **5**, 1613–1622.
- Reddy,M.V. and Randerath,K. (1990) A comparison of DNA adduct formation in white blood cells and internal organs of mice exposed to benzo[*a*]pyrene, dibenzo[*c,g*]carbazole, safrole and cigarette smoke condensate. *Mutat. Res.*, **241**, 37–48.
- Daimon,H., Sawada,S., Asakura,S. and Sagami,F. (1998) *In vivo* genotoxicity and DNA adduct levels in the liver of rats treated with safrole. *Carcinogenesis*, **19**, 141–146.
- Gupta,R.C. (1984) Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. *Proc. Natl Acad. Sci. USA*, **81**, 6943–6947.
- Borchert,P., Wislocki,P.G., Miller,J.A. and Miller,E.C. (1973) The metabolism of the naturally occurring hepatocarcinogen safrole to 1'-hydroxysafrole and the electrophilic reactivity of 1'-acetoxysafrole. *Cancer Res.*, **33**, 575–589.
- Reddy,M.V., Blackburn,G.R., Irwin,S.E., Kommineni,C., Mackerer,C.R. and Mehlman,M.A. (1989) A method for *in vitro* culture of rat Zymbal gland: use in mechanistic studies of benzene carcinogenesis in combination with ³²P-postlabeling. *Environ. Health Perspect.*, **82**, 239–247.
- Li,D., Wang,M., Dhingra,K. and Hittelman,W.N. (1996) Aromatic DNA adducts in adjacent tissues of breast cancer patients: clues to breast cancer etiology. *Cancer Res.*, **56**, 287–293.
- Randerath,K., Putman,K.L. and Randerath,E. (1993) Flavor constituents in cola drinks induce hepatic DNA adducts in adult and fetal mice. *Biochem. Biophys. Res. Commun.*, **192**, 61–68.
- Chen,J.W. and Shaw,J.H. (1996) A study on betel quid chewing behavior among Kaohsiung residents aged 15 years and above. *J. Oral Pathol. Med.*, **25**, 140–143.
- Phillips,D.H., Miller,J.A., Miller,E.C. and Adams,B. (1981) N2 atom of guanine and N6 atom of adenine residues as sites for covalent binding of metabolically activated 1'-hydroxysafrole to mouse liver DNA *in vivo*. *Cancer Res.*, **41**, 2664–2671.
- Van Schooten,F.J., Hillebrand,M.J.X., Van Leeuwen,F.E., Lutgerink,J.T., Van Zandwijk,N., Jansen,H.M. and Kriek,E. (1990) Polycyclic aromatic hydrocarbon–DNA adducts in lung tissue from lung cancer patients. *Carcinogenesis*, **11**, 1677–1681.
- Pfohl-Leszko,A., Grosse,Y., Carriere,V., Cugnenc,P., Berger,A., Carnot,F., Beaune,P. and de Waziers,I. (1995) High levels of DNA adducts in human colon are associated with colorectal cancer. *Cancer Res.*, **55**, 5611–5616.
- Huitfeldt,H.S., Brandtzaeg,P. and Poirier,M.C. (1990) Reduced DNA adduct formation in replicating liver cells during continuous feeding of a chemical carcinogen. *Proc. Natl Acad. Sci. USA*, **87**, 5955–5958.
- Wiseman,R.W., Stewart,B.C., Grenier,D., Miller,E.C. and Miller,J.A. (1987) Characterization of *c-Ha-ras* proto-oncogene mutations in chemically induced hepatomas of B6C3 F1 mouse. *Proc. Am. Assoc. Cancer Res.*, **28**, 147.

Received April 1, 1999; revised August 31, 1999; accepted September 3, 1999