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Safrole–DNA adducts in tissues from esophageal cancer patients: clues to areca-related esophageal carcinogenesis

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Abstract

Epidemiological studies have demonstrated that areca quid chewing can be an independent risk factor for developing esophageal cancer. However, no studies are available to elucidate the mechanisms of how areca induces carcinogenesis in the esophagus. Since the areca nut in Taiwan contains a high concentration of safrole, a well-known carcinogenic agent, we analyzed safrole–DNA adducts by the ^{32}P-postlabelling method in tissue specimens from esophageal cancer patients. In total, we evaluated 47 patients with esophageal cancer (16 areca chewers and 31 non-chewers) who underwent esophagectomy at the National Taiwan University Hospital between 1996 and 2002. Of the individuals with a history of habitual areca chewing (14 cigarette smokers and two non-smokers), one of the tumor tissue samples and five of the normal esophageal mucosa samples were positive for safrole–DNA adducts. All patients positive for safrole–DNA adducts were also cigarette smokers. Such adducts could not be found in patients who did not chew areca, irrespective of their habits of alcohol consumption or cigarette smoking ($p < 0.001$, comparing the areca chewers with non-chewers). The genotoxicity of safrole was also tested in vitro in three esophageal cell lines and four cultures of primary esophageal keratinocytes. In two of the esophageal keratinocyte cultures, adduct formation was increased by treatment with safrole after induction of cytochrome P450 by 3-methyl-cholanthrene. This paper provides the first observation of how areca induces esophageal carcinogenesis, i.e., through the genotoxicity of safrole, a component of the areca juice.

Keywords: Esophageal cancers; Areca; Safrole; DNA adducts

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1. Introduction

Esophageal cancer is an important public health issue worldwide due to its later diagnosis and poorer prognosis than that of other cancers of the gastrointestinal tract. There is a marked variation in incidence of this disease across geographic areas. Although almost all Taiwanese belong to the same ethnic population as Chinese, the annual incidence of esophageal cancer in Taiwan is 6.93 per 100,000, very similar to that in most Western countries [1,2]. This is in sharp contrast to the incidence in certain high-risk areas in Northern China, such as Linxian in Henan Province, where the annual incidence of esophageal cancer exceeds 100 per 100,000 and is the foremost cause of local cancer death [1,2]. Studies in both western and eastern countries have demonstrated that the risk for esophageal cancer is closely related to the consumption of tobacco and alcohol [3–5]. Dietary factors, such as a deficiency in antioxidant vitamins or trace elements or the consumption of pickled vegetables were also significant in affecting the individual risk for esophageal cancer [1,6].

Areca (*Areca catechu*) or betel nut chewing is a common practice in Southeast Asia, especially in India and Taiwan. Previously, we and others have demonstrated that habitual consumption of areca nut could be associated with a higher risk to develop esophageal cancer by acting synergistically with tobacco and alcohol use to increase individual susceptibility to this cancer [3,7–11]. Although betel quid is usually a combination of betel nut, tobacco, *Piper betle* leaf, and slaked lime, the composition of betel quid can vary with geographic location. In Taiwan, tobacco is not included in the preparation of betel quid, and *P. betle* inflorescence or its leaves are added sometimes to betel quid. According to the most recent IARC classification, the areca nut, both with and without addition of tobacco, was classified in Group 1 (carcinogenic to humans) [12]. The *P. betle* inflorescence, which contains a high concentration of safrole (15 mg/g), is used in the preparation of betel quid in Taiwan and Papua New Guinea only [13]. Consequently, chewing betel quid containing *P. betle* inflorescence may contribute to excessive safrole exposure (up to 420 μM in saliva during chewing) in these areas [14]. Safrole (4-allyl-1,2-methylenedioxybenzene) is the major component of the oil of sassafras and a minor constituent of other essential oils and spices, such as anise, basil, nutmeg, mace, and pepper. In the IARC classification, safrole was classified as a Group 2B agent (possibly carcinogenic to humans) [15]. It can induce tumor formation in the liver, lung or oral cavity in rats or mice[16]. Although safrole was not mutagenic in *Salmonella typhimurium* TA98 [17], it induced sister chromatid exchange and micronucleus formation, dose-dependently, in HepG2 human hepatoma cells [18]. The proximate carcinogens of safrole were conjugated by sulfotransferase in liver cytosol to form electrophilic sulfuric acid esters that react with hepatic DNA to give covalently bound adducts [19]. The hepatocarcinogenic potential of safrole is correlated with formation of stable safrole–DNA adducts in target tissue [20–24]. Recently, the presence of safrole-like DNA adducts in oral tissue from oral squamous cell carcinoma patients with a known history of betel quid chewing has been detected by the ^{32}P-postlabelling technique [25]. In addition, the same authors detected the presence of safrole–DNA adducts in primary hepatocellular carcinoma tissue in a patient negative for hepatitis B and C viruses with a 32-year history of betel quid chewing. It was suggested that safrole in betel quid might be an unrecognized risk factor for hepatocellular carcinoma [26]. Given the epidemiological association of areca chewing and the risk for esophageal cancer, studies about how areca induces carcinogenesis in the esophagus are required. This study is the first report on genotoxicity induced by areca in association with esophageal cancer.

2. Materials and methods

2.1. Study population

This study was part of our cohort study of esophageal cancer patients, enrolling newly diagnosed patients at National Taiwan University Hospital from 1996 to 2002 [3,9]. Tissue analysis was performed after informed consent was obtained from each patient. Information on individual environmental exposures was obtained from structured questionnaires. Patients were interviewed by a trained interviewer using a standardized questionnaire. The personal history in the questionnaire of alcoholic beverage consumption, cigarette smoking and areca-nut chewing included the time of

start or quit, duration of consumption, daily amount used, and the type of alcoholic beverage consumed. Cigarette smokers were defined as regular consumers of more than 10 cigarettes per day for at least 6 months. Likewise, areca chewers were defined as regular consumers of areca nut for more than 6 months, and alcohol drinkers as regular consumers of alcoholic beverage more than once a week, for more than 6 months.

2.2. DNA extraction

Forty-seven patients with or without a history of areca chewing and undergoing esophagectomy for esophageal cancer were randomly selected from this cohort. This group included two areca chewers who did not smoke cigarettes. DNA was extracted for carcinogen–DNA-adduct analysis by the standard phenol/chloroform procedure from esophageal cancer tissue and normal esophageal tissue. The DNA concentration was determined by the standard (UV 260) method.

2.3. Detection of safrole–DNA adducts in esophageal cancer tissue and normal esophageal tissue by the ^{32}P-postlabelling technique

The safrole–DNA adduct was detected by the previously described ^{32}P-postlabelling technique [25]. Briefly, 1 μg of DNA was digested in 10 μL 20 mM sodium succinate, 10 mM $CaCl_2$, pH 6.0, containing 5 μg of spleen exonuclease, 5 μg of micrococcal endonuclease at 38 °C for 3.5 h. In order to increase the sensitivity of the assay, the digested deoxyribonucleoside 3′-monophosphates were further treated with 6 μg nuclease P1 in 0.25 M sodium acetate (pH 5.0), 0.3 mM zinc sulfate (pH 5.0) at 37 °C for 40 min. The DNA–carcinogen adducts were then labelled with [γ-^{32}P]ATP (6000 Ci/mmol; Du Pont-NEN, Boston, MA, USA). The labelling procedure was carried out in a buffer mix (100 mM bicine, 100 mM magnesium chloride, 100 mM dithiothreitol, and 10 mM spermidine, pH 9.5) containing 20 μCi of [γ-^{32}P]ATP and four units of T4 polynucleotide kinase (Amersham International). The mixture was incubated at 38 °C for 30 min. The ^{32}P-labelled adducts were resolved on polyethyleneimine (PEI) cellulose TLC plates (Macherey-Nagel or TJ Baker, Germany). The incubation mixture was spotted on a PEI-cellulose TLC plate and developed in a five-directional TLC system as follows. The D1 development was in 1 M sodium phosphate, pH 6.8. The D2 development, in the same direction as D1, was in 3.75 M ammonium formate, pH 3.5. The D3, in the opposite direction relative to D1 and D2, was in 5.3 M lithium formate, 8.5 M urea, pH 3.5. The D4 development, at a right angle to D3, was run in 1.2 M lithium chloride, 0.5 M Tris and 8.5 M urea, pH 8.0. The D5 development was run in the same direction as D4, in 1.7 M sodium phosphate pH 6.0. Adducts were visualized by intensifying screen-enhanced autoradiography using Kodak XAR-5 film. The adduct areas or zones and blank areas were excised from the PEI plate for quantitation by Cerenkov counting. The nature of the adduct was confirmed by mixing a DNA sample with synthesized safrole–dGMP, which was subjected to the same enzymatic digestion followed by development using the above-mentioned conditions, and comparison with patterns derived from individual DNA samples [27]. The profile and location of this adduct is also similar to the DNA product found in 1′-hydroxysafrole-treated HepG2 cells, which has been identified as N^2-(*trans*-isosafrole-3′-yl) 2′-deoxyguanosine [27]. Quantitative estimation of adducts was expressed as number of adducts/10^8 nucleotides. Adduct levels, before and after treatment, were compared by relative adduct labelling (RAL) [21].

2.4. Culture of primary esophageal keratinocytes and cancer cell lines

Normal esophageal keratinocytes were harvested from surgical specimens of normal esophageal mucosa from patients undergoing esophagectomy for esophageal cancer [28,29]. Informed consent was obtained from each patient prior to tissue acquisition. The esophageal mucosa was dissected from the muscular layer with a scalpel. Once harvested, the sample was extensively washed with Dulbecco's modified Eagle medium (DMEM; Gibco, cat. no. 31600-075) in a Petri dish under a laminar-flow cabinet. The specimen was cut into 1 mm sections before being plated. All these procedures were performed following standard sterile protocols. The samples were cultured in medium containing 10% fetal bovine serum (Gibco), 50 μg/ml of gentamicin (Gibco), penicillin (100 μg/ml), and streptomycin (100 μg/ml). The plates were maintained at 37 °C in a humid environment with 5% CO_2. Three esophageal cancer cell lines were also used for in vitro

analysis of safrole–DNA-adduct formation. These cell lines were obtained from tumor tissues from three patients with esophageal squamous cell carcinoma, and had maintained the characteristics of cancer cells including a high N/C ratio, heteroploidy, and regeneration and proliferation of tumor after transplantation into animals [30]. The cancer cell lines or the primary esophageal keratinocytes were co-cultured with the cytochrome-P450 inducer 3-methyl-cholanthrene (MC, 1 μM) for 24 h and then treated with safrole (100 μM) for another 24 h. The cells were then assayed for the presence of safrole–DNA adducts by means of ^{32}P-postlabelling as described above.

3. Results

Tumors and normal adjacent esophageal tissue from 47 cancer patients were evaluated. The clinical profiles of these patients (mean age, 61.4 years) are listed in Table 1. Thirty-nine patients had a diagnosis of squamous cell carcinoma, four had adenocarcinoma, three had spindle-cell sarcoma, and one basaloid squamous cell carcinoma. Seven patients had tumors at the cervical or upper thoracic esophagus, 16 at the mid-thoracic esophagus, and 24 at or middle to lower third or below the lower third thoracic esophagus. Twenty-one patients received neo-adjuvant concurrent chemoirradiation (CCRT) of cisplatin plus 5 FU or paclitaxel and 4000 cGy of irradiation. Fourteen patients chewed betel quid and smoked cigarettes. Two patients chewing areca nut did not smoke cigarettes, while 20 patients smoked cigarettes but did not chew betel quid. Eleven patients did not smoke cigarettes nor chew betel quid. Among the 16 individuals with a history of habitual areca chewing, safrole–DNA adduct formation was detected in one (6%) tumor tissue sample and five (31%) normal esophageal tissue samples. All of the patients in whom adducts were detected were also cigarette smokers. Adducts could not be detected in the cigarette smokers or alcohol drinkers who did not chew betel quid ($n = 24$). The individuals who abstained from all the three substances were also negative for adduct formation in their tissue samples ($n = 4$) (Table 2). Since a high concentration of safrole can be detected in areca quid in Taiwan, we also assayed safrole-induced adduct formation in vitro. Four primary cultures of esophageal keratinocytes and three esophageal cancer cell lines were used for this analysis. Safrole–DNA adducts could be detected in one of the primary esophageal keratinocyte cultures before treatment. In this primary culture, the adduct amount could be increased after induction of cytochrome P450 with MC, followed by treatment with safrole, which increased the RAL from 2.45 to 3.92 (Fig. 2). In another primary culture of esophageal keratinocytes (patient 2 of Fig. 2), the safrole adduct appeared only after safrole treatment following MC induction. In the esophageal cancer cell lines and the other primary esophageal keratinocyte culture, the safrole adducts were undetectable before and after treatment with safrole with or without MC induction. The chromatograms of Figs. 1 and 2

Table 1
Characteristics of the esophageal cancer patients

Age (years) (mean ± S.E.)	61.4 ± 10.8 (40–79)
Sex	
Males	42
Females	5
Cancer type	
Squamous cell carcinoma	39
Adenocarcinoma	4
Other cell types	4
Pathological stage[a]	
Stages 1 and 2	32
Stages 3 and 4	15
Esophageal location	
Upper third	7
Middle third	16
Middle to lower third	5
Lower third	19
CCRT	
Yes	20
No	27
Substance use	
S (+) B (+)	14[b]
S (−) B (+)	2[c]
S (+) B (−)	20[d]
S (−) B (−)	11[e]

CCRT: neoadjuvant concurrent chemoirradiation; S: smoking; B: betel chewing.

[a] TNM staging according to the proposal of Japanese Committee for Registration of Esophageal Carcinoma [39].

[b] Twelve alcohol drinkers, one non-drinker, one unknown.

[c] Two alcohol drinker.

[d] Thirteen alcohol drinkers, five non-drinkers, two unknown.

[e] Four alcohol drinkers, four non-drinkers and three unknown.

Table 2
The levels of safrole–DNA adducts in esophageal cancer patients

	Esophageal cancer patients	Alcohol use (years)	CCRT	RAL (1×10^{-7})
S (+) B (+) ($n = 14$)	6T	27	1	ND
	6N	27	1	8.51
	7N, T	20	1	ND
	11N	8	0	1.7
	12N	10	1	1.6
	15N	46	0	0.55
	22N, T	20	1	ND
	41N	49	0	5.05
	41T	49	0	21.4
	46N	50	1	ND
	55N, T	40	0	ND
	71N, T	0	1	ND
	77N, T	32	0	ND
	78N, T	20	0	ND
	79N, T	28	1	ND
	91N, T	–	1	ND
S (−) B (+) ($n = 2$)	51N, T	8	0	ND
	60N, T	37	1	ND
S (−) B (−) ($n = 11$)	36N, T	–	0	ND
	43N, T	–	0	ND
	45N, T	0	0	ND
	54N, T	0	0	ND
	56N, T	40	0	ND
	59N, T	38	1	ND
	68N, T	18	1	ND
	69N, T	0	0	ND
	76N, T	–	0	ND
	85N, T	38	1	ND
	87N, T	0	1	ND
S (+) B (−) ($n = 20$)	13N, T	0	0	ND
	16N, T	31	0	ND
	17N, T	–	0	ND
	31N, T	46	0	ND
	32N, T	28	0	ND
	33N, T	46	0	ND
	34N, T	52	0	ND
	35N, T	0	0	ND
	40N, T	38	1	ND
	42N, T	0	1	ND
	44N, T	40	1	ND
	47N	0	1	ND
	53N, T	0	0	ND
	61N, T	15	1	ND
	65N, T	16	1	ND
	84N, T	19	1	ND
	86N, T	–	1	ND
	89N, T	31	0	ND
	90N, T	27	0	ND
	98N, T	46	0	ND

N: normal esophageal squamous epithelium; T: tumor tissue; ND: not detectable, below the limit of detection (adducts/10^8 nucleotides); CCRT: neo-adjuvant concurrent chemoirradiation; S: smoking; B: betel chewing and –: status unknown.

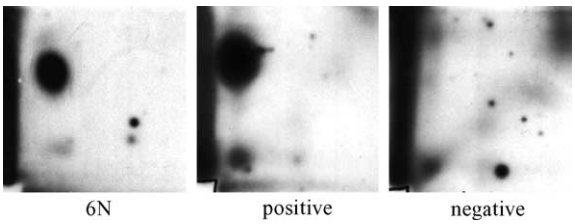

Fig. 1. Example of safroles–DNA adducts detected in the ^{32}P-postlabelling chromatogram from the normal esophageal tissue of one esophageal cancer patient who chewed areca nut (6N), positive: synthesized safrole–dGMP; and control: negative control DNA from a patient who did not chew areca nut.

were developed in PEI-cellulose TLC plates from different manufacturers (Macherey-Nagel or J.T. Baker, Germany, respectively). Furthermore, the pictures for the patients 1 and 2 of Fig. 2 were obtained at different times. These factors made the safrole adduct look different with respect to its position in the chromatograms.

4. Discussion

Our study suggests that areca consumption in Taiwan can induce DNA damage in the esophagus through safrole–DNA adduct formation. This provides the first molecular clue about areca-related carcinogenesis in the esophagus. In our study cohort, there were only two areca chewers who did not smoke cigarettes available for evaluation. The remaining patients chewed areca and were also cigarette smokers ($n = 14$). Five of these 14 areca chewers/cigarette smokers had detectable safrole–DNA adducts in their normal esophageal tissue. These safrole-adducts were not observed in patients who did not chew areca, regardless of whether they smoked cigarettes or drank alcohol. It is also noteworthy that the extent of safrole–DNA adduct formation is not as high as that found in patients with areca-related oral cancer (29/30)[25]. This difference may be attributed to the different exposure patterns to areca juice between the oral cavity and the esophagus in chewing areca. Areca chewing directly and constantly exposes the oral cavity to the areca juice, while the esophagus only briefly encounters the saliva-diluted areca juice during swallowing. DNA-adduct formation induced by areca chewing is, therefore, much more evident in the oral cavity than in the esophagus. However, the differences in adduct formation between the areca

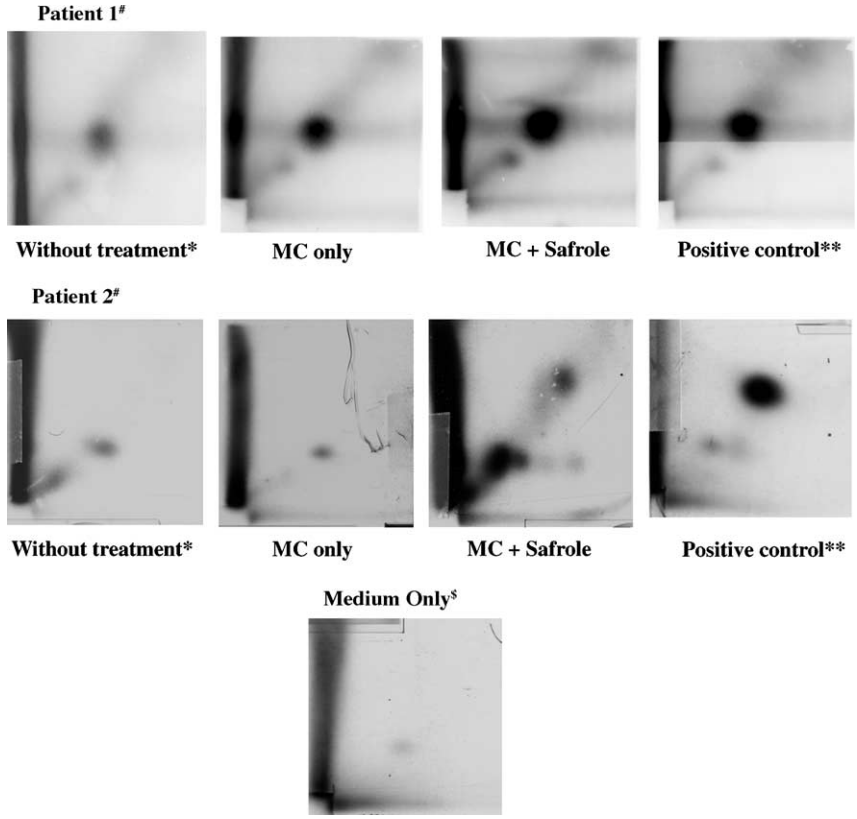

Fig. 2. This figure demonstrates the in vitro assay for safrole-induced DNA-adduct formation in primary esophageal keratinocytes. These cultures were treated with MC (1 μM) for 24 h (MC only), or with MC (1 μM) for 24 h and then with safrole (100 μM) for another 24 h (MC + safrole). Safrole–DNA adduct could be detected in the primary tissue culture of one patient (chewer1) without treatment, but not in that of another patient. The amount of adducts of chewer 1 increased after induction of cytochrome P450 with MC (MC only), and it increased further with subsequent treatment with safrole (MC + safrole). In the other patient (chewer 2), who did not show the safrole adduct in untreated esophageal keratinocytes, the adduct appeared only with treatment of safrole following MC induction (MC + safrole). The increased level of safrole adduct in the MC-treated esophageal keratinocytes of chewer 1 may be attributed to the accelerated biotransformation of endogenous safrole induced by MC. The positions of the safrole adducts in the chromatograms of chewers 1 and 2 look different because they were developed at different times. #From patients with areca consumption. *DNA extracted from the esophageal keratinocytes without any treatment. **Mixture of synthesized safrole–dGMP and DNA from the safrole-treated esophageal keratinocytes after MC induction. $Without adding any sample DNA in the tested medium.

chewers and the non-chewers among the esophageal cancer patients were still evident, suggesting an areca-associated DNA damage in esophageal cancer.

The formation of safrole–DNA adducts requires a two-step biotransformation process. Following initial oxidation of safrole to 1′-hydroxy-safrole by cytochrome P450, it undergoes sulfotransferase-mediated sulfonation to electrophilic sulfuric acid esters that react with DNA to form covalently bound adducts [19]. The activity of various CYP enzymes, including CYP1A, 2E1, 3A, and 4A, can be found in human esophageal mucosa, indicating its capacity to activate safrole into reactive DNA-binding metabolites [31]. The activity of sulfotransferase also exists in different regions of the gastrointestinal tract as demonstrated by their ability to activate several substrates [32]. However, the sulfotransferase activity was lowest in the esophagus among gastrointestinal organs [32], thereby suggesting that sulfotransferase might be the rate-limiting enzyme for the formation of safrole–DNA

adducts. In one of our primary esophageal keratinocyte cultures, which was weakly positive for safrole–DNA adducts, safrole adducts were increased after induction of cytochrome P450 by MC, followed by incubation with safrole. These cells were obtained from a patient with a history chewing areca quid, which might account for the presence of safrole–DNA adducts before treatment. An increase of the safrole–DNA-adduct level after MC induction was also noted, which may be attributed to increased biotransformation of endogenous safrole after cytochrome P450 activation. In the other primary esophageal keratinocyte culture (patient 2 of the Fig. 2), the safrole–DNA adduct appeared only with treatment of safrole following MC induction, it was not detected in the untreated esophageal keratinocyte of this patient. For the other two primary esophageal keratinocytes and the three esophageal cancer cell lines, safrole–DNA-adduct formation was not induced after incubation with safrole, regardless of MC treatment. This variation in the induction of safrole–DNA adducts may be related to inter-individual differences of sulfotransferase activity in the esophagus. For the cells that can metabolize and activate safrole, further induction of cytochrome P450 activity and exposure to safrole could aggravate DNA damage by increasing safrole–DNA adduct formation.

In contrast to the safrole–DNA adduct found in the normal esophageal mucosa, only one of the 16 esophageal cancer patients chewing areca nut had detectable adduct formation in the tumor tissue. This discrepancy in detection rates between the adjacent normal and tumor tissue is similar to the trends of adduct formation found in cancers of the lung, colorectal, breast, or oral cavity [25,33–35]. One possible explanation is that the DNA adducts might be diluted during the process of heterogeneous clonal expansion of cancer cells, thus making the detection more difficult in the tumor tissue than in the normal tissue.

Limitations of our study are that we did not evaluate the markers involved in the other pathways of areca-related carcinogenesis including induction of oxidative stress and alteration of cell growth or differentiation [36–38]. In addition, there were only two patients available who chewed areca nut without consuming tobacco. Most of the patients were simultaneous consumers of tobacco and areca nut. It is possible that tobacco use induces the activation of metabolic enzymes for safrole, and thus influences the formation of safrole–DNA adducts. However, the specific association between the presence of safrole–DNA adducts and the habit of chewing areca nut was not likely to be influenced by the exposure to other environmental toxins; the adducts were not detected in cigarette smokers who did not chew areca. Further study would still be needed to clarify whether the safrole–DNA adduct formation can be induced solely by exposure to safrole-containing areca juice, or whether it needs promotion by tobacco or alcohol exposure.

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