

# Human Exposure to Carcinogenic Heterocyclic Amines and Their Mutational Fingerprints in Experimental Animals

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Heterocyclic amines (HCAs) are mutagens/carcinogens to which humans are exposed on almost a daily basis. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most abundant of the various carcinogenic HCAs (present at a level of 0.56 to 69.2 ng/g of cooked meat or fish), with 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) following it at 0.64 to 6.44 ng/g. HCAs have been found in the urine of healthy people who consume ordinary diets, while patients receiving parenteral alimentation lack, for example, PhIP and MeIQx in their urine. Based on the concentrations of PhIP and MeIQx in urine samples from 10 healthy volunteers, daily intake of MeIQx in Japanese was calculated to be 0.3 to 3.9 µg/person, while that of PhIP was 0.005 to 0.3 µg. The Japanese consume more MeIQx than Americans, whereas Japanese intake of PhIP was about one-third that of Americans. MeIQx-DNA adducts have also been detected in Japanese kidney, colon, and rectum samples using the <sup>32</sup>P-postlabeling method followed by identification using high-performance liquid chromatography (HPLC) analysis; the levels were 0.18, 1.8, and 1.4 per 10<sup>9</sup> nucleotides, respectively. In addition, we elucidated the mutational fingerprints of PhIP by analyzing *Apc* mutations in rat colon cancers induced by this carcinogen. Four of eight tumors had a total of five mutations in the *Apc* gene, four of which featured a guanine deletion from 5'-GTGGGAT-3' sequences. This specific mutation spectrum may be used as a fingerprint of PhIP in evaluating its risk potential for human colon carcinogenesis. Mutations were not found in similar 2-amino-3-methylimidazo[4,5-*f*]quinoline-induced colon lesions. Microsatellite instability was detected in both colon and mammary tumors induced by PhIP. The mechanisms involved in this development of microsatellite instability in PhIP-induced cancers remain to be elucidated. — Environ Health Perspect 104(Suppl 3):497-501 (1996)

Key words: HCA, DNA adducts in human tissues, mutational fingerprint, *Apc*, MI

## Introduction

Heterocyclic amines (HCAs) are major mutagens present in cooked fish and meat to which humans are very regularly exposed. All 10 HCAs so far examined have proved to be carcinogenic in experimental animals,

with target organs including the lung, liver, mammary gland, colon, skin, bladder, endothelium, clitoral gland, forestomach, and hematopoietic system (1,2). The target organs may differ in different animal

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Abbreviations used: HCAs, heterocyclic amines; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3':2'-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3':2'-*d*]imidazole; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; HPLC, high-performance liquid chromatography; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; B[a]P, benzo[*a*]pyrene; RER<sup>+</sup>, replication error<sup>+</sup>; MI, microsatellite instability; HNPCC, hereditary nonpolyposis colorectal cancers, TLC, thin-layer chromatography.

species and sometimes even in different strains within the same species (3).

Epidemiological studies have demonstrated that people who eat much heavily browned meat are at a 2.0- to 6.0-fold higher risk of colorectal cancer development. A large intake of heavily browned gravy increased the risk further to 6.41 (4,5). These foodstuffs are major sources of HCAs, and information on human exposure levels to HCAs appears to be of basic importance for prevention of cancer. We have therefore measured urinary excretion levels of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), which are the most abundant HCAs in cooked meat and fish. We also measured DNA adduct levels of these two compounds in human samples and compared them with adduct levels of other environmental carcinogens.

Some chemicals leave specific mutational fingerprints. For instance, aflatoxin B<sub>1</sub> induces a unique type of mutation in the p53 gene, a G to T transversion at the third position of codon 249. UV radiation also induces characteristic types of mutations in the p53 gene. Thus, we also analyzed whether HCAs leave fingerprints in their induced tumors.

## Results and Discussion

### Levels of Human Exposure to HCAs

HCAs are produced by heating amino acids (3-amino-1,4-dimethyl-5*H*-pyrido[4,5-*b*]indole [Trp-P-1], 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole [Trp-P-2], 2-amino-6-methyldipyrido[1,2-*a*:3':2'-*d*]imidazole [Glu-P-1], 2-aminodipyrido[1,2-*a*:3':2'-*d*]imidazole [Glu-P-2]), protein (2-amino-9*H*-pyrido[2,3-*b*]indole [AαC], 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole [MeAαC]), or proteinaceous foods such as fish and meat (HCAs including an imidazole ring, such as 2-amino-3-methylimidazo[4,5-*f*]quinoline [IQ], 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline [MeIQ], MeIQx, and PhIP) (6).

Analysis of HCAs in daily foods revealed PhIP to be the most abundant of the various carcinogenic species; it was present at a level of 0.56 to 69.2 ng/g of cooked meat or fish, with MeIQx following at 0.64 to 6.44 ng/g (6).

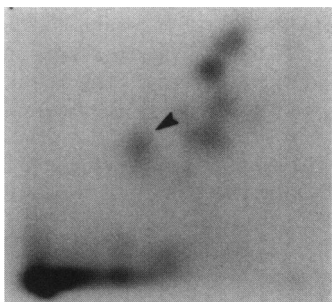
We measured urinary excretion levels of PhIP and MeIQx in 10 healthy volunteers (7). The ranges (ng/24 hr urine) were 0.12 to 1.97 and 11 to 47 for PhIP and MeIQx,

respectively. Urinary excretion of orally ingested MeIQx in unchanged form has been reported to be 1.2 to 4.3% and that of PhIP to be 0.6 to 2.3% (8). Based on these excretion rates, daily intake by the Japanese volunteers was calculated to be 0.3 to 3.9  $\mu\text{g}/\text{person}$  for MeIQx and 0.005 to 0.3  $\mu\text{g}/\text{person}$  for PhIP. No significant difference was found between smokers and nonsmokers. This is as expected since PhIP is not detectable in cigarette smoke (9).

Recently, Skog et al. (10) reported that dietary exposure from 100 g of meat and cooking-pan residues is 0 to 3.0  $\mu\text{g}$  MeIQx and 0.2 to 9.5  $\mu\text{g}$  PhIP based on food analysis. Layton et al. (11) estimated average daily intake of people in the United States to be 16.6 ng/kg/day PhIP and 2.6 ng/kg/day MeIQx based on 3-day dietary records of 3,563 individuals; they estimated the intake of A $\alpha$ C to be 5.17 ng/kg/day (11). Although MeIQx values in our data and Swedish data were comparable, PhIP intake by American and Swedish people seems to be higher than Japanese. The significance of the relatively high intake of A $\alpha$ C remains unclear because it induced liver cancer and hemangioendothelial sarcomas but not colon cancer in CDF<sub>1</sub> mice that are considered to be a resistant strain in colon carcinogenesis; it also caused a very high level of *lacI* mutations in the colon epithelium of *lacI* transgenic mice (C57Bl/6 $\times$ SWR) (12). Thus a possible contribution to colon carcinogenicity cannot be precluded.

### DNA Adduct Levels

MeIQx- and PhIP-DNA adduct levels in human organs were analyzed by the <sup>32</sup>P-postlabeling method, with modifications (13). DNA was digested with spleen phosphodiesterase and micrococcal nuclease, and



**Figure 1.** TLC pattern of MeIQx-DNA adduct ( $1.4/10^9$  nucleotides) formation in the human colon. The arrowhead indicates a spot corresponding to deoxyguanosin-8-yl-MeIQx 5'-monophosphate. TLC was performed as described by Tada et al. (13).

modified nucleotides were phosphorylated with T4 polynucleotide kinase under adduct-intensification conditions. The reaction products were digested briefly with nuclease P1 to remove 3'-phosphate and then dinucleotides or oligonucleotides were converted to mononucleotides with phosphodiesterase I. There was no appreciable reduction in yields of both DNA adducts either by treatment with nuclease P1 (4  $\mu\text{g}/13 \mu\text{l}$ , 37°C, 10 min) or phosphodiesterase I (60 mU at pH 9.0, 37°C, 30 min). After thin-layer chromatography (TLC) development, materials in the spots corresponding to guanine-C8-MeIQx and guanine-C8-PhIP were extracted with 0.5 ml of 4M pyridinium formate (pH 4.5), and their identities were confirmed using high-performance liquid chromatography (HPLC) with a TSK-ODS-80Ts column with an acetonitrile-phosphate buffer developing system (Figures 1 and 2).

DNAs were isolated from liver, kidney, colon, pancreas, lung, and heart samples from five human autopsy cases. DNAs were also isolated from surgical specimens of normal parts of the colorectum (four), liver (two), and kidney (two) of patients having cancers in the same organs. MeIQx adducts were detected in the kidney of an autopsy sample and in the rectum and colon of surgical samples; their levels were 0.2, 1.4, and 1.8 per  $10^9$  nucleotides,

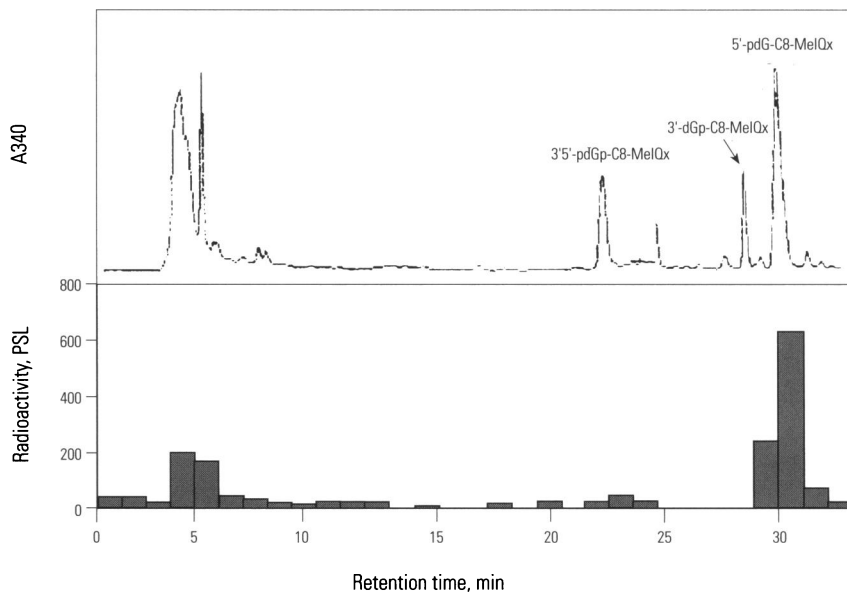
respectively. No PhIP-DNA adducts were detected in any of these surgical samples examined (14).

Recently, Friesen et al. (15) found PhIP-DNA adducts in two of six colon mucosa samples of Americans, with a level of about 3 adducts/ $10^8$  nucleotides. These results are in good agreement with the generally high intake of PhIP by American people, and their HCA adduct findings are comparable to those for 4-hydroxy-1-(3-pyridyl)-1-butanone released from the adduct of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and DNA in peripheral lung tissue and the tracheobronchus (16) (Table 1). A similar benzo[*a*]pyrene (B[*a*]P)-DNA adduct level has also been reported (18) and approximately 10 times more 4-aminobiphenyl-DNA adducts have been described (17). It is thus very plausible that HCAs play some role(s) in human carcinogenesis.

### Mutational Fingerprints of HCAs

Some carcinogens leave unique mutational fingerprints in their induced tumors (19); this may be very useful for evaluating the significance of HCAs in human carcinogenesis.

To elucidate whether characteristic HCA-associated mutations exist, we investigated genetic alterations in rat colon tumors caused by HCAs, Glu-P-1, IQ, and



**Figure 2.** HPLC pattern of MeIQx-DNA adduct formation in the human colon. A pooled extract of the adduct spot from 10 plates was applied to a TSK gel ODS-80Ts column (5  $\mu\text{m}$  particle size, 4.6 $\times$ 250 mm, Tosoh Corp., Tokyo, Japan) with the following solvent system: 0 to 10 min, 10% acetonitrile in 25 mM phosphate buffer (pH 2.0); 10 to 70 min, a linear gradient at 10 to 50% acetonitrile in 25 mM phosphate buffer (pH 2.0) with a flow rate of 1 ml/min at the ambient temperature.

PhIP. Seven colon adenocarcinomas induced by Glu-P-1, 11 adenocarcinomas induced by IQ, and 9 adenocarcinomas induced by PhIP were examined for *ras* family gene mutations. Only one Glu-P-1-induced colon tumor had a *K-ras* mutation (codon 12, GGT to GTT) (20), and no mutations were detected in the *N-ras* or *Ha-ras* genes of any of these tumors. Further, no p53 gene mutations were detected in any of these tumors although 60 to 70% of human colon cancers have mutations in the p53 gene (21).

The *APC* gene is mutated in 60% or more of sporadic human colon tumors (22), and usually mutations occur at a very early stage of colon carcinogenesis. To allow analysis of mutations of the rat *Apc* gene in HCA-induced colon tumors, we first determined its cDNA sequence and genomic structure. The rat *Apc* cDNA coding region shows a high homology with its human counterpart, being 86.2% identical at the nucleotide level and 90.2% at the amino acid level (23). Its mRNA was found to be derived from 15 exons as in the case human *APC* mRNA, and the exon-intron boundary structure was also conserved (24). We determined the intron sequences flanking the exons and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis was performed using colon tumor DNAs (Table 2). Four of eight colon tumors had mutations in the *Apc* gene. Since two mutations were detected in one of these tumors, the total number of mutations was five. All of these tumors featured a guanine deletion from 5'-GGGA-3' sequences; two were at codon 635, one at codon 869, and the remaining two were at codon 1413. Further, four of the five shared a common seven-nucleotide target sequence, 5'-GTGGGAT-3'. In the rat *Apc* gene, this 5'-GTGGGAT-3' sequence is present at only two sites where codons 635 and 1413 are included, and two mutations were detected at each of these two sites (24). Because PhIP forms DNA adducts by covalent binding mainly at guanine-C8 (25,26), these mutations might be directly induced by the PhIP-DNA adduct.

In contrast to PhIP, *Apc* mutations were detectable in only 2 of 13 colon tumors induced by IQ (24). Since these two mutations were T to C and C to T mutations at codons 523 and 921, respectively, and given the fact that IQ also forms DNA adducts mainly with guanine bases (27), there is a possibility that these mutations were not directly induced by the

**Table 1.** DNA adduct levels in human tissues.

Carcinogen	Organ	Adducts/nucleotide × 10 <sup>9</sup>	Detection method	References
MeIQx	Rectum	1.8	<sup>32</sup> P-postlabeling	(14)
	Sigmoid colon	1.4		
	Kidney	0.2		
PhIP	Colon	29.0	GC-MS- <sup>32</sup> P-postlabeling	(15)
NNK	Lung (smoker, n = 9)	ND-16.3 (3.7) <sup>a</sup>	GC-NICI-MS	(16)
	Tracheobronchus (smoker, n = 4)	ND-10 (5.3)		
	Lung (smoker, n = 8)	ND-2.1 (0.3)		
	Tracheobronchus (nonsmoker, n = 4)	ND-0.9 (0.3)		
4-ABP	Lung (smoker)	ND-495	GC-NICI-MS	(17)
	Lung (nonsmoker)	14.1		
	Bladder	ND-39.4		
B[a]P	Lung (n = 21)	ND-80 (9.5)	<sup>32</sup> P-postlabeling	(18)

Abbreviations: ND, not detected; GC-NICI-MS, gas chromatography-negative ion chemical ionization-mass spectrometry; B[a]P, benzo[a]pyrene; GC-MS, gas chromatography-mass spectrometry; 4-ABP, 4-aminobiphenyl. <sup>a</sup>Figures in parentheses indicate average values.

**Table 2.** *Apc* gene mutations in rat colon tumors induced by PhIP and IQ.

Sample	Codon	<i>Apc</i>	
		Mutation	Result
PhIP-2-1	635	GGTGGGATA → GGTGGATA	Frameshift
PhIP-13	635	GGTGGGATA → GGTGGATA	Frameshift
PhIP-17	1413	AGTGGGATT → AGTGGATT	Frameshift
PhIP-18-4	869	TCCGGGAAC → TCCGGAAC	Frameshift
	1413	AGTGGGATT → AGTGGATT	Frameshift
IQ-3-1	523	GGCTGCATG → GGCCGCATG	Cys → Arg
IQ-1-2-2	921	GCACGA → GCATGA	Arg → Stop

**Table 3.** Microsatellite instability in colon and mammary tumors induced by PhIP in comparison with other carcinogens.

Cancer	Carcinogen	MI positive/examined	Tumor number	Loci altered
Colon	PhIP	7/8	PhIP-2-1	<i>PBC2</i>
			PhIP-13	<i>PRLR, SMST</i>
			PhIP-17	<i>PND, ADRB2, PRLR</i>
			PhIP-18-4	<i>APOC3</i>
			PhIP-2-2	<i>FGG, ADRB2</i>
	IQ	0/9	PhIP-7	<i>PPY</i>
			PhIP-18-1	<i>IGHE</i>
			MT-1	<i>D3Mgh9, D9Mit3, TNF, D20Mgh1</i>
			MT-24	<i>D6Mgh3, D20Mit1, PND</i>
			MT-23	<i>MT1PA, DXMgh1, TNF</i>
Mammary gland	PhIP	9/15	MT-2	<i>D2N91, D16Mgh3</i>
			MT-15	<i>RBP-2, D6Mgh7</i>
			MT-20	<i>D2Mit2</i>
			MT-1	<i>TAT</i>
			MT-3	<i>REN</i>
	DMBA	2/12	MT-18	<i>D14Mgh2</i>
			MT-6	<i>D15Mgh4</i>
			MT-8	<i>D18Mgh3</i>

DMBA, 7,12-dimethylbenz[a]anthracene.

carcinogen. The 5'-GTGGGAT-3' sequence around codon 635 is also conserved in the human *APC* gene, and future analysis of *APC* mutations in human colon cancers

developing in persons consuming large quantities of heavily browned meat may therefore provide us with important information as to etiology.

Microsatellite instability (MI) is observed in virtually all of human hereditary nonpolyposis colorectal cancers (HNPCC) (28). At least 70% of HNPCC patients were proven to have mutations in one of four mismatch repair enzymes, hMSH2 (MutS-related), hMLH1, hPMS1, and hPMS2 (MutL-related) and number of microsatellite alterations per total amount of analysis exceeded 20% in these RER<sup>+</sup> (replication error<sup>+</sup>) HNPCC (29). MI has also been observed in 10 to 20% of sporadic colorectal cancers. Examination of 85 simple sequence repeats on 19 chromosomes revealed seven of eight PhIP-induced colon tumors to have mutations in at least one locus (30). Three tumors had mutations in

more than one locus. In contrast, no microsatellite mutations were detected in IQ-induced colon tumors. Further, MI was observed in PhIP-induced rat mammary cancers as summarized in Table 3. The microsatellite mutation rates observed in PhIP-induced colon and mammary cancers were about 2%, and much lower than that observed in RER<sup>+</sup> human colon cancers demonstrated to have mutations in mismatch repair enzymes (30).

At least three mechanisms can be considered for the PhIP-induced MI. One is direct induction of mismatch repair enzyme mutations by the carcinogen. Mutations of *GTBP* are known to result in a low rate of MI (31). A second possibility is functional

impairment of mismatch repair enzymes; this speculation is supported by the fact that the colon and mammary cancers were induced by continuous feeding of PhIP. Third, MI might have been caused by covalent binding of PhIP to simple sequence repeats. However, the simple repeat sequence composed of (AAAT)<sub>n</sub> was also mutated. Thus, the probability of the third mechanism being important is low.

Although the involved mechanisms remain to be elucidated, MI can be considered as characteristic for PhIP. More detailed studies are needed to use MI as a mutational fingerprint of PhIP with the aim of determining the causes of human cancers.

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