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Detection of 2-amino-1-methyl-6-phenylimidazo [4,5-b]-pyridine (PhIP)–DNA adducts in human pancreatic tissues

J. ZHU[1], A. RASHID[2], K. CLEARY[2], J. L. ABBRUZZESE[1], H. FRIESS[3], S. TAKAHASHI[4], T. SHIRAI[4], & D. LI[1]

Departments of [1]Gastrointestinal Medical Oncology and [2]Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA, [3]University of Bern, Inselspital, Bern, Switzerland, [4]Nagoya City University Medical School, Nagoya, Japan

Abstract
Recent epidemiological investigations have observed an association between the consumption of grilled or barbecued meat and an increased risk of pancreatic cancer, suggesting that dietary exposure to heterocyclic aromatic amines (HCA) may contribute to the development of this disease. 2-Amino-1-methyl-6-phenylimidazo [4,5-b]-pyridine (PhIP) is the most abundant HCA found in well-done and grilled meats. To determine whether HCA-induced DNA damage is present in the human pancreas, immunohistochemistry and computer-assisted image analysis were used to measure PhIP–DNA adducts in 54 normal pancreatic tissues (N) from persons without pancreatic cancer and in 38 normal adjacent pancreatic tissues (A) and in 39 cancer tissues (T) from 68 patients with pancreatic adenocarcinoma. PhIP–DNA adducts were detected in 53 N, 34 A and 39 T samples. Mean values ($\pm$SD) of the absorbency for PhIP staining were 0.22 ± 0.04, 0.24 ± 0.04, and 0.24 ± 0.03 for N, A, and T samples, respectively ($p = 0.004$). Using the median absorbency (0.21) of the samples from normal controls as the cut-off, 71% of A and 77% of T tissues, compared with 48% of N tissues, were distributed in the higher range ($p = 0.009$). The odds ratio of pancreatic cancer was 3.4 (95% confidence interval 1.5–7.5, $p = 0.002$) for individuals with a higher level of PhIP–DNA adducts. This is the first report of the detection of PhIP–DNA adducts in human pancreatic tissue samples obtained from patients with unknown exposure to HCA. Although limited by the small sample size, these preliminary results suggest that PhIP exposure may contribute to human pancreatic cancer development.

Keywords: *DNA adduct, 2-amino-1-methyl-6-phenylimidazo [4,5-b]-pyridine (PhIP), pancreatic cancer, immunohistochemistry*

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Introduction

Pancreatic cancer is the fourth leading cause of cancer death in both men and women in the USA (American Cancer Society 2005). Because diagnosis usually occurs late in the natural history of the tumour, the mortality rate of pancreatic cancer is

Correspondence: D. Li, Department of Gastrointestinal Medical Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 426, Houston, TX 77030, USA. Tel: +713-834-6690. Fax: +713-834-6153. E-mail: dli@mdanderson.org

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approximately equal to its incidence rate; about 86% of patients will die during the first year following diagnosis, and by 5 years after diagnosis virtually all patients have died (Washaw & Fernandez-del Castillo 1992). The 30 000 Americans who die of this disease annually account for approximately 5% of all cancer deaths in the USA (Niederhuber et al. 1995). Very little is known about the aetiology of pancreatic cancer, which makes its prevention almost impossible. Novel approaches are needed to identify the genetic and environmental determinants of pancreatic cancer risk.

Cigarette smoking is the only established environmental risk factor for pancreatic cancer. Cigarette smoke contains a variety of chemical carcinogens. Tobacco carcinogens and carcinogen-induced DNA adducts have been detected in human pancreatic juice and pancreatic tissues, respectively, providing direct evidence that the pancreas is a target tissue for tobacco carcinogens. (Wang et al. 1998, Thompson et al. 1999, Prokopczyk et al. 2002). Another important risk factor associated with pancreatic cancer seems to be the diet. Generally speaking, increased risks have been associated with the consumption of animal protein and fat and decreased risks with the intake of vegetables, fruits, and dietary fibres (Lyon et al. 1993, Ji et al. 1995, Ohba et al. 1996, Gold & Goldin 1998). The diet is an important source of carcinogen exposure. For example, nitrosamines, heterocyclic aromatic amines (HCA), and polyaromatic hydrocarbons can be derived either from natural food or during the process of food preparation. Several epidemiological studies have shown an association between the consumption of smoked meat, grilled or barbecued meat, fried food, and dehydrated or preserved food and an increased risk for pancreatic cancer (Ghadirian et al. 1995, Ji et al. 1995, Anderson et al. 2002). These studies raise the question of whether dietary exposure to nitrosamines, HCAs, and other carcinogens is responsible for pancreatic cancer.

HCAs are formed by the pyrolysis of amino acids and creatine or creatinine when meat is cooked at high temperatures (Jagerstad et al. 1991, Skog et al. 1995, Schut & Snyderwine 1999). 2-Amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) is the most abundant dietary HCA. In animal studies, long-term exposure to PhIP has induced tumours in the mammary gland in female rats and in the colon in male rats (Ito et al. 1991, Imaida et al. 1996), and these organs are the two most common sites of carcinogenesis in Western countries. Epidemiology studies have shown that dietary HCA exposure is associated with an increased risk of human colon and breast cancers (Zheng et al. 1998, Sinha et al. 2000, Butler et al. 2003).

The PhIP–DNA adduct level was highest in the pancreas of male rats after a single oral administration of PhIP compared with colon, lung, heart, and liver, indicating that the pancreas was highly susceptible to HCA exposure (Kaderlik et al. 1994), and dietary administration of HCAs induced pancreatic carcinogenesis in hamsters and rats (Yoshimoto et al. 1999, Hirose et al. 2000). PhIP is bioavailable in human populations, as demonstrated by its detection in human urine (Ushiyama et al. 1991, Kidd et al. 1999, Kulp et al. 2000) and milk (DeBruin et al. 2001). Furthermore, PhIP–DNA adducts have been detected by accelerator mass spectrometry in human breast tissue samples obtained from individuals given dietary equivalent levels of ^{14}C-labelled PhIP (Lightfoot et al. 2000) and by immunohistochemical analysis in breast tissues from women with unknown exposure to HCA (Zhu et al. 2003). To test the hypothesis that dietary HCA exposure may increase the risk of human pancreatic cancer, the present study used immunohistochemical analysis to determine the presence of PhIP–DNA adducts in the pancreatic tissue of patients with or without

pancreatic cancer. A higher level of PhIP–DNA adducts was found in pancreatic tissues from patients with pancreatic cancer than in tissues from those without this cancer.

Materials and methods

Frozen or paraffin sections of pancreatic tissue samples were obtained from three sources: the NCI Cooperative Human Tissue Network (Midwestern Division), The University of Texas M. D. Anderson Cancer Center Tissue Bank, and an organ transplant unit at the University of Bern, Bern, Switzerland. Surgical samples of 39 pancreatic adenocarcinomas and 38 normal adjacent tissues were obtained from the NCI Tissue Network and the tissue bank of the M. D. Anderson Cancer Center. The 77 tissue samples included nine pairs of tumour and normal adjacent tissues from the same patients, thus the total number of cancer patients included in this study was 68. Fifty-four surgical or autopsy samples of normal pancreatic tissues were collected from 44 patients with conditions other than pancreatic cancer, such as gunshot wound, heart disease, and non-pancreatic malignancies (NCI Tissue Network), and from ten organ donors. The only information available for organ donors and for patients donated tissue samples to the NCI Tissue Network was age, race, and sex. Exposure information, such as cigarette smoking, was available for the 37 patients treated at the M. D. Anderson. The M. D. Anderson Cancer Center Institutional Review Board approved the study.

Paraffin sections were prepared from all frozen tissue samples. PhIP–DNA adducts were detected using an immunohistochemical method as previously described (Zhu et al. 2003). Briefly, the paraffin-embedded sections were baked at 65°C overnight, deparaffinized in xylene, and rehydrated in serial alcohol. In addition, endogenous peroxidase activity was blocked using 1% H_2O_2 in methanol for 20 min. After treatment using RNase and pepsin, the sections were blocked using 3% bovine serum albumin and normal goat serum. Next, the primary anti-PhIP–DNA adduct polyclonal antibody was incubated with the sections at 4°C overnight in a humid chamber at a dilution of 1:3000. In addition, the biotinylated secondary antibody was incubated with the sections at 37°C for 30 min, at a dilution of 1:200. The antibody complex was detected using an avidin-biotin-peroxidase complex solution and visualized using 3,3′-diaminobenzidine (Zymed Laboratories, Inc., San Francisco, CA, USA). A negative control was included in each experiment by omitting the primary antibody.

Tissue sections showing nuclear staining in at least 10% of the epithelial cells were considered to be positive. PhIP–DNA adduct levels were semi-quantified by measuring the absorbency of nuclear staining in three to five randomly selected fields in each positive section. The stained nuclei of ductal epithelial cells per sample were captured as grey-scale images from three randomly selected low-power fields (100 ×) and the staining intensities were semi-quantified via image analysis. Additionally, the nuclear staining intensity was expressed as absorbency. About 1200 cells of each sample were evaluated. Empirically, it was found that most positively stained nuclei were in the range of 0–170 on the 0–225 grey scale. Thus, a threshold of 0–170 was set consistently for all sections analysed to exclude cells with negative or very low staining intensities. To reduce analytical bias, each batch of samples included samples

obtained from both cancer and control patients, and the case-control status was unknown to the person who performed the assays.

The mean values of each tissue sample were used to calculate the mean ($\pm$SD) of the PhIP–DNA adduct absorbency in different types of tissues. The mean absorbency was compared in different types of tissue as a continuous variable using the Student's t-test. The median values of the PhIP–DNA adduct absorbance were compared by the non-parametric Mann–Whitney U-test. Two-tailed p-values were calculated to determine statistical significance; the level of significance was set as $p < 0.05$. The absorbance values were analysed as categorical variables using the median value of controls as the cut-off. Logistic regression was applied to calculate the odds ratio (OR) and 95% confidence interval (CI) for the association between PhIP–DNA adducts and pancreatic cancer risk with adjustment for age, sex, and race.

Results

The mean ages of the 68 cancer patients and 54 pancreatic cancer-free controls were 57.0 ± 13.4 and 54.8 ± 15.8 years, respectively ($p = 0.402$). There were no significant differences in the sex and racial distributions between cancer patients and controls. There were 41 men (60.3%) and 27 women (39.7%) among cases and 25 men (46.3%) and 39 women (53.7%) among controls ($p = 0.12$). Non-Hispanic whites, Hispanics, African-Americans, and Asian Americans accounted for 83.8, 7.4, 7.4 and 1.5% of the cases and 85.2, 0, 13.0 and 1.9% of the controls, respectively ($p = 0.17$).

PhIP–DNA adducts were analysed in 54 normal tissue sections from the pancreatic cancer-free controls, 29 sections of normal adjacent pancreatic tissues from persons with pancreatic cancer, and 30 sections of pancreatic adenocarcinoma, as well as nine paired sections of normal adjacent tissue and pancreatic adenocarcinoma from the same patients. The presence of PhIP–DNA adducts in the pancreas was shown as nuclear staining detected by immunohistochemistry (Figure 1). Although many fibroblasts in the stroma and islet cells showed nuclear staining, the intensity was mostly weak. In most sections, the islets of Langerhans were absent. Thus, quantitative analysis was focused on the staining of pancreatic epithelial cells only.

Positive staining was found in 53 (98%) of 54 normal pancreatic tissues of controls, 34 (89%) of 38 normal adjacent pancreatic tissues, and 100% of 39 pancreatic cancer tissues (Table I). Age, sex, and race had no significant effect on the level of PhIP–DNA adducts (data not shown). There was no significant difference in the PhIP–DNA adduct levels between the normal adjacent pancreatic tissues and cancer tissues: the mean $\pm$SD/median (range) of the absorbency were $0.24 \pm 0.04/0.24$ (0–0.33) and $0.24 \pm 0.03/0.24$ (0.17–0.31), respectively. However, these PhIP–DNA adduct levels were significantly higher than those in the controls [$0.22 \pm 0.04/0.21$ (0–0.32), $p = 0.004$ and 0.022, respectively]. Using the median value (0.21) of the absorbency in the control patients as the cut-off, it was found that 71 and 77% of normal adjacent tissues and cancer tissues but only 48% of control tissues were distributed in the higher range ($p = 0.009$). There was no significant difference in the absorbency between the normal adjacent and cancer tissues in all study subjects (Table I) or in the nine paired normal and tumour tissues from the same patients (Table II). Thus, a mean value was calculated from each of the nine pairs of tissues and all tissues from cancer patients were pooled together. The association between the levels of PhIP–DNA adducts and the risk of pancreatic cancer was then estimated in the 54 controls

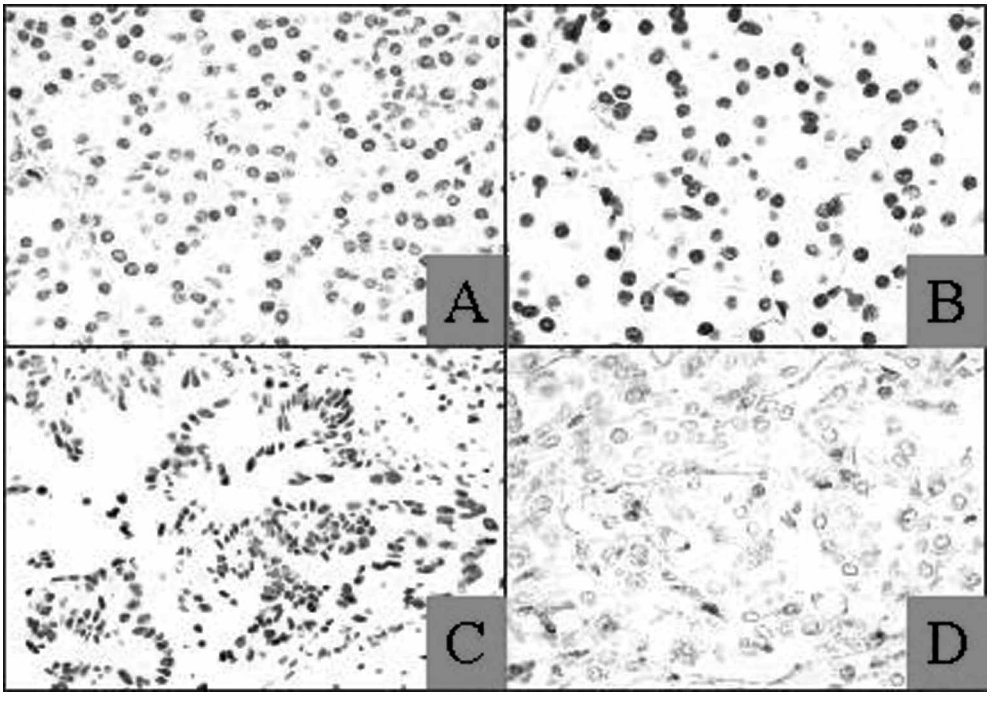

Figure 1. Nuclear staining of a PhIP–DNA adduct in normal pancreatic tissue of a control patient (A), adjacent pancreas tissues of patients with pancreatic cancer (B, D), and pancreatic adenocarcinoma (C). Negative staining is shown in (D). Original amplification: A, B and D, ×400; C, ×200.

and 68 cancer patients. Logistic regression analysis of the dichotomized absorbance values demonstrated that the adjusted OR of pancreatic cancer was 3.4 (95% CI 1.5–7.5, $p=0.002$) for individuals with a higher level of PhIP–DNA adducts after adjustment for age, sex, and race (Table I).

Table I. PhIP–DNA adducts and the risk of pancreatic cancer.

Variable	Normal tissues ($n=54$)	Adjacent tissues ($n=38$)	Tumour tissues ($n=39$)	p
Positive staining	53 (98%)	34 (89%)	39 (100%)	
Absorbance (continuous):				
Mean ± SD[a]	0.22 ± 0.04	0.24 ± 0.04	0.24 ± 0.03	0.004[b]
Median (range)	0.21 (0–0.32)	0.24 (0–0.33)	0.24 (0.17–0.31)	0.022[c]
Absorbance (categorical):				
≤0.21	28 (52%)	11 (29%)	9 (23%)	
>0.21	26 (48%)	27 (71%)	30 (77%)	0.009[d]
Adjusted OR (95% CI)	1.0	3.4 (1.5–7.5)	0.002[e]	

[a]Positive samples only.
[b]Analysis of variance (ANOVA).
[c]Non-parametric test for the median.
[d]χ^2-test.
[e]Logistic regression analysis of 54 normal controls and 68 cancer patients with adjustment for age, sex and race.

Table II. Paired normal adjacent pancreatic tissues and tumour tissues.

Samples	Age (years)	Sex	Race	Absorbance	
				Normal	Tumour
MDACC 01	54	male	white	0.26	0.20
MDACC 02	64	female	white	0.26	0.27
MDACC 03	73	female	white	0.27	0.22
MDACC 04	53	female	other	0.17	0.21
MDACC 05	53	male	white	0.20	0.27
MDACC 06	66	male	white	0.17	0.24
PNCI-83	54	male	white	0.23	0.24
PNCI-84	71	male	white	0.22	0.23
PNCI-97	31	female	white	0.24	0.28
Mean $\pm$ SD				0.22 ± 0.04	0.24 ± 0.03

$p = 0.34$ by ANOVA.

Information on smoking history was available for 37 cases. In both normal adjacent and tumour tissues, never smokers had a higher level of adducts than did ever smokers (Table III).

Discussion

The paper reported on the detection of PhIP–DNA adducts in the pancreatic tissues of patients having or not having pancreatic cancer, as well as a significant association between the levels of PhIP–DNA adducts in such tissues and the risk of pancreatic cancer. To the authors' knowledge, this is the first study to demonstrate the presence of PhIP–DNA adducts in pancreatic tissues from patients without preplanned exposure to PhIP. The present data provided evidence supporting the hypothesis that exposure to PhIP may contribute to the aetiology of human pancreatic cancer.

Several laboratories have attempted to measure PhIP–DNA adducts in human tissues or lymphocytes, but the established gas chromatography/electron capture mass spectrometry (GC/MS) and ^{32}P-post-labelling methods showed a low or no probability of detecting DNA adducts in white blood cells and tissues of colon, pancreas, and bladder (Friesen et al. 1994). In rats treated with PhIP at 0.1 μg kg^{-1} day^{-1}, a dose of PhIP approximating to a human daily intake, PhIP–DNA adducts could not be detected (Friesen et al. 1996). Although accelerated mass spectrometry did detect PhIP–DNA adducts in breast tissues of humans given dietary equivalent levels of PhIP labelled with ^{14}C before surgery (Lightfoot et al. 2000), this method cannot be applied to epidemiological studies in human populations naturally exposed to HCA.

Table III. PhIP adducts by smoking status.

Tissue type	Never smokers		Ever smokers		p
	n	Mean $\pm$ SD	*n*	Mean $\pm$ SD	
Normal Adjacent	7	0.26 ± 0.05	12	0.22 ± 0.08	0.158
Tumour	7	0.27 ± 0.01	11	0.24 ± 0.03	0.032
Both	14	0.27 ± 0.03	23	0.23 ± 0.06	0.027

The availability of the antibody against PhIP–DNA adducts has provided a novel alternative method for detecting PhIP–DNA adducts in human tissues (Takahashi et al. 1998). In the present authors' previous study, the specificity and sensitivity of the immunohistochemical method was determined in a human mammary epithelial cell system and a clear dose–response relationship was demonstrated in nuclear staining intensities of cells exposed to different doses of N-hydroxy PhIP (Zhu et al. 2003). The detection limit of the immunohistochemical method seemed to be in the range of one adduct per 10^5–10^7 nucleotides. A mean staining intensity of 0.10 approximately corresponded to 2.6 adducts/10^7 nucleotides as detected by ^{32}P-post-labelling in that experimental system. In the same study, PhIP–DNA adduct levels were measured in human breast tissues and a positive association was observed between the level of PhIP adducts and the risk of breast cancer.

The current study employed the same immunohistochemical method and detected PhIP–DNA adducts in a high percentage of normal pancreatic tissues obtained from cancer patients and controls as well as in the cancer tissues. The high positive rates could be explained by the fact that human exposure to PhIP is quite common. Specifically, PhIP has been detected not only in cooked fish and meat, but also in cigarette smoke (Manabe et al. 1991), in urban air and diesel engine exhaust particulate (Manabe et al. 1992), and in beer and wine (Manabe et al. 1993). Because of the limited amount of tissue samples available for this study, the results could not be verified by using other biochemical method in the same samples. Nevertheless, the present authors' previous study in a cell system and in human breast tissues has demonstrated a high concordance of results from the immunohistochemical method and the ^{32}P-post-labelling method. Furthermore, the primary antibody against PhIP–DNA adducts has been shown not to cross-react with unmodified DNA or with DNA modified by other carcinogens (Takahashi et al. 1998). It is unlikely that the high positive rate observed in the present study was an artefact. Future studies using different methods in the same samples will be required to confirm further the present observations. Using semi-quantitative analysis, a significantly higher level of PhIP–DNA adducts was found in the cancer patients than in the control patients. Furthermore, a significant association between the levels of PhIP–DNA adducts in pancreatic tissues and the risk of pancreatic cancer was observed. Although limited by the small sample size, this observation has provided supporting evidence for an association between HCA exposure and human pancreatic cancer.

HCAs consist of many components (Jagerstad et al. 1991, Skog et al. 1995, Schut & Snyderwine 1999), and which one is responsible for the increased risk of pancreatic cancer is unknown. Using a specially designed meat preparation questionnaire, epidemiological studies have shown a connection between the amounts of DiMeIQx (2-amino-3,4,8-trimethylimidazo [4,5-f]quinoxaline) and PhIP consumed through the diet and an increased risk for human colon and breast cancers (Zheng et al. 1998, Sinha et al. 2000, Butler et al. 2003). In animal models, DiMeIQx has been shown to induce invasive cancer of the pancreas in hamsters (Yoshimoto et al. 1999), and PhIP was found to be a weak pancreatic carcinogen, targeting acinar cells and inducing acidophilic foci in rats (Hirose et al. 2000). Whether PhIP is the HCA compound responsible for human pancreatic cancer needs further investigation.

Due to the difficulties in obtaining pancreatic tissue samples for research, tissue samples were used from different sources. Although the cancer patients and controls were well matched by age, sex, and race in the present study, the major limitation of

the current study was the lack of exposure information on both dietary HCA intake and cigarette smoking. As an exposure marker, the higher level of PhIP–DNA adducts detected in cancer patients compared with controls could be due to differences in dietary habits or smoking history between the two groups. Although the current study observed a significantly lower level of PhIP adducts in ever smokers compared with never smokers in a limited number of cancer cases, these results need to be interpreted cautiously. It is possible that these observations were made by chance alone. Another possibility is that smoking has a mild effect on the level of PhIP–DNA adducts in the pancreas and this mild effect could be masked by the predominant effect of dietary HCA exposure. The results of the current study indicated the presence of PhIP-induced DNA damage in the human pancreas, but the exact relationship between HCA exposure and the risk of pancreatic cancer needs further epidemiological investigation. The authors are current conducting a molecular epidemiological study investigating the associations between dietary HCA exposures, genetic susceptibility to such exposure, and the risk of pancreatic cancer. It is hoped that this study will help in the clarification about whether PhIP is a novel target for the primary prevention of pancreatic cancer.

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