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Expression of cytochrome P450 1A1/2 and 3A4 in liver tissues of hepatocellular carcinoma cases and controls from Taiwan and their relationship to hepatitis B virus and aflatoxin B_1– and 4-aminobiphenyl–DNA adducts

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Cytochrome P450 enzymes play a major role in the metabolism of several of the chemical carcinogens involved in the development of hepatocellular carcinoma (HCC). To investigate by immunohistochemistry interindividual differences in these enzymes, polyclonal antisera and immunoperoxidase staining were used to detect the expression of CYP1A1/2 and 3A4 in 37 surgical control tissues and 105 tumour and adjacent non-tumour tissues of HCC cases from Taiwan. There was variability in the expression and staining pattern for both CYP1A1/2 and 3A4 in all tissue types. In tissues from controls, there was no correlation between P450 expression and smoking history or hepatitis B virus antigen status. Since these samples had been previously analysed for the DNA adducts of aflatoxin B_1 (AFB_1), a dietary mould contaminant, and 4-aminobiphenyl (4-ABP), a component of cigarette smoke, we also investigated the relationship between P450 levels and DNA adducts. 4-ABP–DNA adducts were higher in tissues with elevated levels of CYP1A1/2 ($p = 0.02$). Overall there was no relationship between CYP1A1/2 or CYP3A4 and AFB_1–DNA adducts in control tissues. Staining intensity for CYP1A1/2 and 3A4 followed the order: tumour tissues < control tissues < adjacent non-tumour tissues. CYP1A1/2 levels tended to be lower in tumour and adjacent non-tumour tissues than for CYP3A4. In HCC cases, 4-ABP–DNA adducts were higher in subjects with higher levels of CYP1A1/2, stratified by tissue type, but these differences were not significant. For CYP3A4, in contrast to control tissues, there was a significant association with AFB_1–DNA adducts in tumour and adjacent non-tumour tissue of HCC cases. These results suggest that one factor influencing carcinogen–DNA adducts is levels of specific P450 enzymes. However, adduct formation *in vivo* is a complex processes dependent upon numerous genetic and environmental factors.

Keywords: carcinogen–DNA adducts, cigarette smoking, immunohistochemistry.

Abbreviations: 4-ABP, 4-aminobiphenyl; AFB_1, aflatoxin B1; CYP1A1/2, cytochrome P450 1A1/1A2; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; P450, cytochrome P450; PBS, phosphate buffered saline; SD, standard deviation.

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Introduction

Hepatocellular carcinoma (HCC) is one of the major cancers in the world and the leading cancer for males in Saharan Africa and southern Asia including Taiwan, Thailand, and southern China (Beasley 1982, IARC 1992). It has a very poor prognosis and a limited response to current therapies. Two major environmental factors have been implicated in the aetiology of HCC, hepatitis B virus (HBV) infection and exposure to the chemical carcinogen, aflatoxin B_1 (AFB_1) (Harris and Sun 1984, IARC 1994). A synergistic interaction between those two agents in the development of HCC has been reported (Sell *et al*. 1991, Ross *et al*. 1992, Wang *et al*. 1996). Several studies using immunohistochemical methods found 50–70% of liver tissues from HCC patients in Taiwan had detectable levels of AFB_1–DNA (Zhang *et al*. 1991, Ross *et al*. 1992, Lunn *et al*. 1997). In addition to these risk factors, studies in animals as well as human epidemiological studies have suggested that cigarette smoking is also related to HCC (Chen *et al*. 1991, 1993). To obtain further information on the role of smoking as a risk factor for HCC, we previously measured DNA adducts resulting from 4-aminobiphenyl (4-ABP), a carcinogen present in cigarette smoke, in liver tissue of HCC cases and controls. These studies found higher levels of 4-ABP–DNA in cases compared with controls, providing further evidence for a role for smoking in HCC (Wang *et al*. 1998). In animals, a linear relationship has been reported between levels of the major guanine C8 adduct of 4-ABP in liver DNA and liver tumour incidence (Poirier *et al*. 1995).

Most chemical carcinogens, including AFB_1 and 4-ABP, require metabolic activation to reactive intermediates before they can bind to DNA or protein. Cytochrome P450-dependent mono-oxygenases (P450) are important enzymes in these phase I reactions and are responsible for the metabolism of numerous endogenous and exogenous compounds (Guengerich and Shimada 1991, Landi *et al*. 1999). The major P450 enzymes involved in the metabolic activation of chemical carcinogens are in the CYP 1, 2, and 3 families (Guengerich and Shimada 1991, Kawajiri and Fujii-Kuriyama 1991). CYP1A1 is involved in the conversion to reactive intermediates of numerous polycyclic aromatic hydrocarbons including benzo(*a*)pyrene while CYP1A2 metabolically activates arylamine carcinogens such as 4-ABP and heterocyclic amines derived from cooked beef (Guengerich and Shimada 1991). While several P450s including CYP1A2 metabolize AFB_1, CYP3A4 is believed to be principally responsible for its bioactivation in human liver (Guengerich and Shimada 1991, Guengerich *et al*. 1998). P450 subtypes have different distributions in the various organs and some, including CYP1A1/2 and 3A4, are inducible (Lewis 1996, Chang *et al*. 1997). Marked interindividual variation in the expression of P450 genes at the mRNA and protein level have been reported (McKinnon and McManus 1996, Anderson *et al*. 1998), yet the genetic basis for this variability is still not clear.

Chronic HBV infection is one of the most important risk factors for HCC (IARC 1994). In HBV transgenic mice expressing the HBsAg and x gene, a marked induction of specific P450 was observed (Kirby *et al*. 1994). For this reason, altered carcinogen metabolism in the presence of hepatitis-induced liver injury has been hypothesized as one mechanism for the synergistic interaction between infection with HBV and exposure to chemical carcinogens (Wild *et al*. 1993).

Immunohistochemistry provides the sensitivity, specificity, and spatial resolution required for identifying particular cell types containing individual P450

Table 1. Characteristics of HCC cases and controls from Taiwan.

	Cases (%)	Controls (%)	*p* value
Gender[a]			
Male	96 (92.3)	17 (47.2)	
Female	8 (7.7)	19 (52.8)	<0.001
Smoking status[b]			
Non-smoker	46 (50.6)	22 (68.8)	
Smoker	45 (49.4)	10 (31.2)	0.075
HBsAg			
Negative	26 (24.8)	28 (75.7)	
Positive	79 (75.2)	9 (24.3)	<0.001
AFB_1–DNA[c]			
Nondetectable	21 (20.0)	21 (56.8)	
Detectable	84 (80.0)	16 (43.2)	<0.0001
4-ABP–DNA[d]			
Low (≤0.187)	13 (12.4)	18 (48.7)	
High (>0.187)	92 (87.6)	19 (51.4)	<0.001

[a] Data on sex missing from one case and one control.
[b] Smoking status missing on 14 cases and five controls.
[c] Detectable adducts in either tumour or non-tumour tissue of a subject; detection limit ~1 adduct/10^6 (Zhang *et al*. 1991).
[d] Relative staining intensity, arbitrary units; values for cases are mean of tumour and non-tumour tissue when both data were available. Data were missing for 4 tumours and 11 non-tumour tissue.

forms. It also requires minimal amounts of sample and can be applied to stored paraffin blocks. In this study, we used immunohistochemical methods to investigate levels of CYP1A1/2 and 3A4 in tumour and adjacent non-tumour tissues of HCC cases and normal tissue of surgical controls and their relationship to smoking and HBV status. We also determined the relationship between P450 levels and the DNA adducts of AFB_1 and 4-ABP which were previously reported in the same tissues studied here (Zhang *et al*. 1991, Lunn *et al*. 1997, Wang *et al*. 1998).

Materials and methods

Study population

The population consisted of 105 pathologically confirmed HCC cases (mean age 54.7 ± 15.8 years) and 37 surgical controls (mean age 58.1 ± 17.1) who were patients at the National Taiwan University Hospital between 1984 and 1995; this poopulation has been described previously (Lunn *et al*. 1997, Wang *et al*. 1998). Informed consent was obtained from patients and the study was approved by the appropriate institutional review committee. Clinical–pathological characteristics and smoking status were obtained from hospital charts. Table 1 summarizes previously reported data on gender, smoking status, HBsAg, and DNA adducts for the HCC cases and controls (Lunn *et al*. 1997, Wang *et al*. 1998). There were significant differences between cases and controls for gender ($p<0.001$) and HBsAg status ($p<0.001$) with more male and HBsAg positive cases than controls. AFB_1– and 4-ABP–DNA adducts were also higher in cases than controls. Four normal USA liver samples were obtained from Columbia-Presbyterian Medical Center patients who died from heart diseases.

Immunohistochemistry for detection of CYP1A1/2 and 3A4 on paraffin-embedded sections

Staining was carried out with personnel blinded to prior DNA adduct data. The goat anti-CYP1A1/2 and 3A4 antisera were from GENTEST Corp. Woburn, MA (catalogue nos 210105 and 24249, respectively). The 3A4 antisera has some cross-reactivity with CYP3A5. Paraffin-embedded sections (5 μm) of liver tissue, fixed in buffered formalin, were deparaffinized then, to increase assay sensitivity, heated using a 400 watt microwave oven for 10 min in 10 mM citric acid, pH 6.0. To quench endogenous peroxidase activity, slides, after washing with PBS, were incubated with 0.3% H_2O_2 in methanol at room temperature for 20 min. Non-specific binding was blocked with 1.5% normal rabbit serum (Vector Laboratories, Burlingame, CA) and slides were incubated with CYP1A1/2 and 3A4

antisera at 1:100 dilution in 1.5 % normal rabbit serum overnight at 4 °C. ABC staining was performed using Vectastain kits (ABC Elite Kit, Vector Laboratories). After dipping the slides in 1 % Triton X-100 (Mallinckrodt, Paris, KT) in PBS for 30 s, slides were dehydrated and cleaned in serial ethyl alcohol and xylenes and mounted with permount (Fisher Scientific, Pittsburgh, PA). Counter staining was carried out with Harris haematoxylin and ethyl green.

As a control, USA liver tissues which consistently gave positive and negative results for CYP1A1/2 and 3A4, were stained with each batch of slides. Staining levels were analysed either qualitatively or quantitatively. For the qualitative analysis, a combined score was used. Immunostaining intensity of the centrilobular area was graded 1 to 3 (1, negative or weak; 2, intermediate; 3, high). The percent positive cells was graded on a scale of 1 to 3 (1, 10 % hepatocytes positive; 2, 10–50 % positive; 3, > 50 % positive). A combined score was obtained by adding the staining intensity score to the percent positive score (1–2, low; 3–4, intermediate; 5–6, high). Relative staining intensity of normal liver tissues from the USA autopsy and Taiwan surgical controls was also quantitated using a Cell Analysis System (CAS) 200 microscope (Becton Dickinson, San Jose, CA) using the Cell Measurement Program. Five centrilobular (zone 3) areas were randomly chosen and 10 cells from each area quantitated for a total of 50 cells. Data are expressed as the average optical cytoplasmic density.

Western blot

To validate the immunohistochemical method for human CYP1A1/2 and 3A4 detection, Western blot analysis was carried out on the four USA liver samples for which large amounts of material were available. Tissues (30 % w/v) were homogenized at 4 °C with a polytron in KCl–PBS buffer (0.1 M KCl, 1mM EDTA in PBS) then centrifuged at 15 000 *g* for 2 h. The supernatant was centrifuged at 105 000g for 1 h and the microsomal pellet resuspended in 10 mM Tris buffer (pH 7.4, 0.1 mM EDTA, 20 % glycerol). Protein concentration was determined with a BCA kit (Pierce, Rockford, IL) and 10 μg run on 12.5 % acrylamide gels. Proteins were transferred to a nitrocellulose filter for 2 h at 70 volts in 25 mM Tris–HCl, pH 8.0, 195 mM glycine and 10 % methanol. Filters were blocked with 5 % BSA in PBS for 1 h, then goat anti-CYP1A1/2 or 3A4 antisera were added (1:500 in PBS containing 0.5 % skim milk) for 1 h. After washing, filters were incubated with peroxidase-conjugated affinity purified rabbit anti-goat IgG secondary antiserum (1:4000) (Jackson Immunoresearch Laboratories Inc., West Grove, PA) for 1 h, then washed. Chemiluminescence was visualized using an ECL kit (Amersham Life Science, Piscataway, NJ). Band intensities were quantitated on a Molecular Dynamics Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

The Taiwan samples were previously analysed for AFB_1– and 4-ABP–DNA adducts, and HBsAg using immunoperoxidase techniques (Zhang *et al*. 1991, Lunn *et al*. 1997, Wang *et al*. 1998). The prior AFB_1–DNA adduct data are dichotomous with samples classified as positive or negative. The 4-ABP–DNA immunoperoxidase staining was quantitated by the CAS200 microscope and data are expressed as a continuous variable measuring relative intensity of nuclear staining. The CYP1A1/2 and 3A4 data were log-transformed to obtain a more normal distribution. The means differences of log-transformed data, grouped by smoking and HBV status among controls, were tested by *t*-test. Fisher's exact test was used to examine the associations between DNA adducts and levels of CYP1A1/2 and 3A4 expression in tumour and non-tumour tissues of HCC cases and normal tissues of controls. All analyses were carried out using Statistical Analysis System software.

Results

In both the USA and Taiwan control liver tissues there was a gradient in CYP1A1/2 and 3A4 immunostaining within the liver acinus. Representative staining for the Taiwan controls are shown in figure 1 ((A) and (B) for CYP1A1/2 and 3A4, respectively). Positive staining was concentrated throughout the cytoplasm. The strongest staining was in hepatocytes in zone 3 (perivenular, centrilobular) of the liver acinus, with much weaker staining seen in zone 2 (midzonal) and zone 1 (periportal). In the control tissues from Taiwan, there was a 2–3-fold variation in staining for CYP1A1/2 and 3A4 (data not shown).

To compare the immunohistochemistry method with Western blot, both methods were carried out on the four USA livers for which large amounts of tissue were available. In this small sample, there was a 2–3-fold range in staining intensity

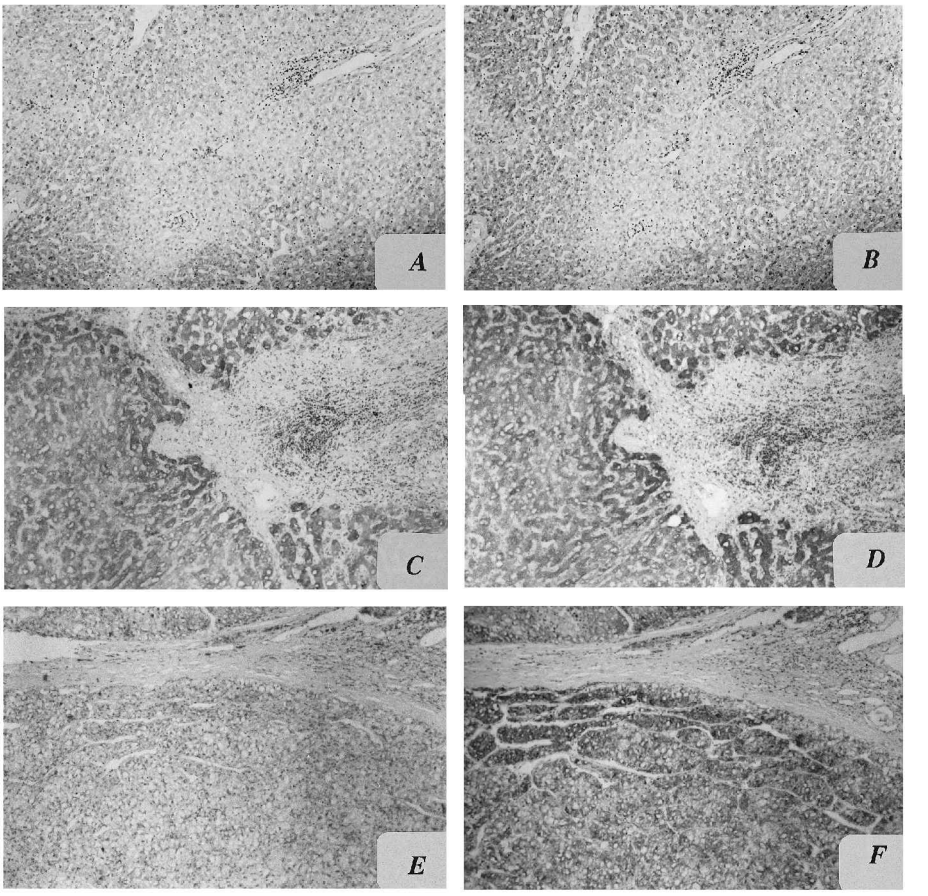

Figure 1. Representative immunoperoxidase staining for: CYP1A1/2 (A) and 3A4 (B) of a formalin-fixed, paraffin-embedded section of a Taiwan control liver tissue. Immunoreactivity is predominantly present within the cytoplasm of hepatocytes located around the central vein (zone 3, centrilobular); CYP1A1/2 (C) and 3A4 (D) expression in an adjacent non-tumour tissue of an HCC case. Diffuse cytoplasmic immunostaining in all hepatocytes of the cirrhosis area is observed with a similar staining pattern and intensity for both enzymes; negative staining for CYP1A1/2 (E) in the tumour tissue of an HCC case and diffuse, strong positive staining for 3A4 (F) in all tumour cells of the same case. (A) and (B) were from the same subject, (C) and (D) from another and (E) and (F) from a third. The nuclei have been counterstained with Harris haematoxylin. ×100

by both Western blot and immunohistochemistry with both antisera and good correlations between both methods of quantitation ($r^2 = 0.91$ for CYP1A1/2 and 0.78 for CYP3A4 (data not shown). The reproducibility of staining was also investigated by repeat staining of a single sample for both proteins. The daily within sample coefficient of variation (CV), counting 50 cells per sample, ranged from 16 to 22 % for CYP1A1; repeat analysis of the same sample on separate days had a CV of 4 % ($n = 3$). Similarly for CYP3A4, the within sample CV was 13–15 % and the repeat analysis CV was 6 %.

The relationship between levels of CYP1A1/2 and 3A4 and smoking and HBV status was investigated in control tissues from Taiwan (table 2). Levels of CYP1A1/2 were lower in smokers compared with non-smokers and higher in

Table 2. Relationship between log-transformed relative immunoperoxidase staining intensity for CYP1A1/2 and 3A4 levels and smoking, HBV status, and DNA adducts in Taiwanese control liver tissues[a].

	Number	CYP1A1/2		CYP3A4	
		Mean (SD)	*p* value	Mean (SD)	*p* value
Smoking status[b]					
Non-smoker	22	−1.340 (0.195)		−1.299 (0.195)	
Smoker	10	−1.508 (0.244)	0.05	−1.469 (0.330)	0.16
Serum HBsAg status					
Negative	28	−1.414 (0.216)		−1.363 (0.232)	
Positive	9	−1.265 (0.289)	0.08	−1.242 (0.388)	0.40
AFB_1–DNA[c]					
Non-detectable	21	−1.390 (0.271)		−1.335 (0.313)	
Detectable	16	−1.362 (0.201)	0.73	−1.331 (0.232)	0.97
4-ABP–DNA[d]					
Low (≤0.187)	19	−1.464 (0.180)		−1.405 (0.228)	
High (>0.187)	18	−1.287 (0.266)	0.02	−1.258 (0.310)	0.11

[a] Data modified from Lunn *et al*. (1997) and Wang *et al*. (1998).
[b] Data on smoking missing for five controls.
[c] Detectable adducts in either tumour or non-tumour tissue of a subject; detection limit ~1 adduct/10^6 Zhang *et al*. (1991).
[d] Relative staining intensity, arbitrary units; values for cases are mean of tumour and non-tumour tissue when both data were available. Data were missing for 4 tumours and 11 non-tumour tissues.

HBsAg positive subjects ($p = 0.05$ and 0.08, respectively). CYP1A1/2 was higher in subjects with elevated levels of 4-ABP–DNA adducts ($p = 0.02$) but there was no relationship with AFB_1–DNA. For CYP3A4, there was no relationship with smoking, HBsAg status or adducts.

Because of the large amount of time required to obtain quantitative data on P450 immunostaining and the known alterations of P450 levels in tumour tissues, only qualitative information (low, medium and high as detailed in Materials and Methods) was obtained on case samples. Control tissues were also analysed with the qualitative method to compare the two techniques. Although there were few subjects in several categories, in general there was good agreement between both methods of analysis with test for trend p values of < 0.0007 and 0.0004 for CYP1A1/2 and 3A4, respectively.

In the HCC cases, the distribution of CYP1A1/2 and 3A4 in tumour and adjacent non-tumour tissues was different from that of control tissues. Normal liver structure was not present even in adjacent non-tumour tissue and the pattern of CYP1A1/2 and 3A4 expression was patchy in appearance and diffuse. For each subject, the intensity and pattern of CYP1A1/2 and 3A4 staining was similar in adjacent non-tumour tissues (figure 1, (C) and (D) respectively). In tumour tissue, stronger staining was observed for CYP3A4 compared with CYP1A1/2 (figure 1, (E) and (F) respectively). There was widespread immunoreactivity for CYP1A1/2 and 3A4 in most well- and moderately differentiated HCC tissues but not in poorly-differentiated tumours.

Table 3 gives the qualitative data on both enzyme levels in all tissues. Tumour tissues had lower levels of both CYP1A1/2 and 3A4 than non-tumour or control tissues. Eighty-three percent of tumours had low levels of CYP1A1/2 compared with 0% and 5.4% of non-tumour and control tissues, respectively. The difference between tumour and control tissues, when numbers in the low category were

Table 3. Qualitative immunoperoxidase staining for CYP1A1/2 and 3A4 for the tumour, adjacent non-tumour and control tissues.

	Tumour tissue, *N* (%)	Non-tumour tissue, *N* (%)	Control tissue, *N* (%)
CYP1A1/2			
Low	88 (83.8)	–	2 (5.4)
Intermediate	9 (8.5)	56 (53.3)	34 (91.9)
High	1 (0.1)	40 (38.1)	1 (2.7)
Unknown	7 (6.6)	9 (8.6)	–
CYP3A4			
Low	27 (25.7)	–	2 (5.4)
Intermediate	63 (60.0)	28 (26.7)	30 (81.1)
High	9 (8.6)	68 (64.8)	5 (13.5)
Unknown	6 (5.7)	9 (8.6)	–

Table 4. Relationship between qualitative immunoperoxidase staining for CYP1A1/2 levels and quantitative immunoperoxidase staining 4-ABP–DNA adducts in tumour and non-tumour tissues from HCC cases.

	Tumour tissue[a] Mean ± SD (*N*)	Non-tumour tissue[b] Mean ± SD (*N*)
Low	0.308 ±0.116 (86)	–
Intermediate	0.258 ± 0.081 (8)	0.281 ± 0.103 (52)
High	0.356 (1)	0.301 ± 0.106 (39)

[a] 4-ABP–DNA data missing on four tumour tissues.
[b] 4-ABP–DNA data missing on 11 non-tumour tissues.

Table 5. Relationship between qualitative immunoperoxidase staining for CYP1A1/2 and 3A4 and AFB_1–DNA adducts in tumour and non-tumour tissues from HCC cases.

	AFB_1-DNA Adducts			
	Tumour tissue		Non-tumour tissue[a]	
	Detectable (%)	Non-detectable (%)	Detectable (%)	Non-detectable (%)
CYP1A1/2				
Low	57(64.8)	31 (35.2)	–	–
Intermediate	9 (100)	–	20 (35.7)	36 (64.3)
High	1 (100)	–	21 (52.5)	19 (47.5)
CYP3A4				
Low	6 (22.2)	21 (77.8)	–	–
Intermediate	53 (84.1)	10 (15.8)	6 (21.4)	22 (78.6)
High	8 (88.9)	1 (11.1)	35 (51.5)	33 (48.5)

[a] AFB_1–DNA adduct data missing on four non-tumour tissues.

compared with the combined intermediate and high, was significant ($p = 0.001$). For CYP3A4, 25.7% of tumours had low levels compared with 0% and 5.4% of non-tumour and control tissues, respectively. Comparing P450 levels of tumour and adjacent non-tumour samples from the same subject, lower levels of CYP1A1/2 were observed in tumour compared with non-tumour tissues. Non-tumour tissues tended to have higher levels of staining than control tissues.

The mean relative immunoperoxidase staining for 4-ABP–DNA for each tertile of CYP1A1/2 staining intensity in case tissues is given in table 4. In adjacent non-

tumour tissues, there was a general trend towards higher adducts in tissues with higher CYP1A1/2 levels but this relationship was not significant.

For AFB_1–DNA adducts, there was a trend toward higher frequency of detectable adducts in subjects with higher P450 levels (table 5). If low CYP1A1/2 subjects were compared with intermediate and high subjects combined, the association between detectable adducts and higher P450 was significant ($p = 0.03$) in tumours. CYP3A4 expression was significantly associated with AFB_1–DNA adducts in tumour tissues ($p < 0.001$) when samples with low levels were compared with a combination of those with intermediate and high levels. For non-tumour tissue this relationship was also significant ($p < 0.007$) when comparing samples with intermediate to those with high expression.

Discussion

A number of enzymes including members of the cytochrome P450 family are involved in human hepatic metabolism of AFB_1, 4-ABP, and other chemical carcinogens involved in the development of HCC. Measurement of these enzymes can be carried out by Western blot, immunohistochemistry, and *in situ* hybridization as well as enzyme activity (Yokose *et al.* 1989, McKinnon *et al.* 1991, Murray *et al.* 1992, McKinnon and McManus 1996, Yamazaki *et al.* 1997, Anderson *et al.* 1998). In this study, because of the limited amount of tissue available from the HCC cases and controls, immunohistochemical methods were used to investigate CYP1A1/2 and 3A4 expression in paraffin sections. These data were combined with prior data on HBV antigens and AFB_1– and 4-ABP–DNA adducts to investigate relationships between P450 expression, HBV status, and DNA damage levels.

Consistent with prior immunohistochemistry (Yokose *et al.* 1989, Murray *et al.* 1992) and mRNA studies (McKinnon and McManus 1996), both CYP1A1/2 and 3A4 were preferentially localized in zone 3 hepatocytes of the liver acinus in normal liver tissue. The zonal heterogeneity in P450 expression has been suggested to arise from altered gene transcription or mRNA stabilization rather than translational or post-translational mechanisms [reviewed in McKinnon and McManus (1996)]. It was also suggested that this may be partly related to the varied circulatory micro-environment of hepatocytes in the different acinus zones. A 15–60-fold range in CYP1A2 mRNA or protein levels has been found in control liver samples in other studies [reviewed in Landi *et al.* (1999)]. But, using the immunoperoxidase method, only a 2–3-fold interindividual variation in CYP1A1/2 and 3A4 expression was found in normal tissues in this study. This may be due to the small number of samples assayed or some limitations of the immunoperoxidase method. For example, microwave treatment may not have fully unmasked all the epitopes for antibody binding. Tumour and adjacent non-tumour tissue of HCC cases also showed interindividual variation in staining intensity. Variable expression of both CYP1A and 3A in primary malignant liver tumours by Western blot (Kirby *et al.* 1993), immunohistochemistry (Murray *et al.* 1993) and *in situ* hybridization (McKinnon *et al.* 1991) has been observed previously.

The expression of P450s in tumour and adjacent non-tumour tissue of HCC is different from that in normal or hepatitis liver tissue. The staining pattern observed in this study, a diffuse cytoplasmic immunoreactivity for tumour tissues, was similar to that found in a previous study (Murray *et al.* 1993). Both tumour and

adjacent non-tumour tissues showed loss of normal lobular structure. The higher expression of P450s in adjacent non-tumour tissues than in tumour tissue may be due to a larger number of more proliferative hepatocytes and has been observed previously (Kirby *et al.* 1993). This higher expression was manifested by both a stronger staining in individual cells and a more dispersed staining throughout the liver sections.

CYP1A2 is constitutively expressed in human liver and inducible by cigarette smoking and other exposures [reviewed in Landi *et al.* (1999)]. There is conflicting evidence on the expression of CYP1A1 in human liver. Using *in situ* hybridization and quantitative PCR techniques, the presence of CYP1A1 mRNA has been established [reviewed in McKinnon and McManus (1996)]. But several laboratories have not been able to demonstrate the presence of CYP1A1 protein, suggesting that if present, it is at very low levels. A more recent paper using a highly specific antibody against CYP1A1 and a sensitive chemiluminescence system, has detected levels ranging from 0.4 to 5 pmol CYP1A1 mg^{-1} microsomal protein (Drahushuk *et al.* 1998). This can be compared with levels of 40–100 pmol mg^{-1} reported for CYP1A2 (Yamazaki *et al.* 1997). The commercial antiserum used in the present report was generated against rat liver CYP1A1 and cross-reacts with both human CYP1A1 and1A2 proteins. Thus, we were concerned about whether the immunoreactivity measured in the tissue samples resulted from CYP1A1 or 1A2 protein. After completing the studies reported here with the GENETEST Corp. antibody, we obtained the CYP1A1 specific antibody used by Drahushuk *et al.* (1998) (generous gift of Dr Conney, H. Gelboin, National Cancer Institute) and tested a subset of liver tissues. Very low levels of staining were observed suggesting that the variation in staining observed with the CYP1A1/2 antibody was due to CYP1A2.

We also investigated the relationship between HBV infection and cigarette smoking on P450 expression. While smoking is a well established inducer of CYP1A1/2 in some individuals, we observed no increase in CYP1A1/2 or 3A4 smokers. Tissue samples were obtained at the time of surgery at which point subjects had been hospitalized for variable time periods. The time since diagnosis was even longer with estimates of 1–3 weeks. During this time frame, cigarette smoking patterns likely decreased and may have influenced the results. Studies in humans have demonstrated that induction of various P450 lasts less than 1 week [reviewed in Conney (1986)].

HBsAg carrier state has been associated with changes in the activity of cytochrome P450 (Geubel *et al.* 1987). However, another investigation did not find an association between viral presence and CYP1A2 function as measured by the ^{14}C-caffeine breath test (Horsmans *et al.* 1995). Nor is HBx alone sufficient to induce P450 alterations in transgenic mice (Chomarat *et al.* 1998). In the present study, no correlation between CYP1A1/2 or 3A4 expression and HBsAg status was observed.

4-ABP–DNA adducts levels in liver tissues were significantly related to expression of CYP1A1/2 in control tissues. Two prior studies have obtained similar results using an *in vivo* phenotyping assay for CYP1A2 activity and comparing data to 4-ABP–haemoglobin adducts (Bartsch *et al.* 1990, Landi *et al.* 1996). Higher levels of *N*-oxidation capacity were associated with higher levels of 4-ABP–haemoglobin adducts. In the present study, no relationship was observed between CYP1A1/2 and AFB_1–DNA adducts in control tissues. This also is in

agreement with prior data suggesting that CYP1A2 is protective against AFB_1-induced tumours in rodents. This may be due to the preferential formation of detoxification products by this enzyme [reviewed in Guengerich *et al.* (1998)].

While *in vitro* multiple human P450s, including the 1A, 2A, 2B, and 3A gene families, metabolize AFB_1, CYP3A4 is believed to be principally responsible for formation of the reactive *exo* epoxide *in vivo* (Shimada and Guengerich 1989, Forrester *et al.* 1990, Crespi *et al.* 1991, Guengerich *et al.* 1998). In the present study, there was a significant correlation between CYP3A4 expression and AFB_1–DNA adducts in tumour and adjacent non-tumour tissues but not in control tissues. These results suggest that CYP3A4 may be more important in DNA adduct formation in tissues from cases than controls. A single previous study examined CYP3A4 expression in HCC samples and found detectable levels in 12 (38.7%) (Murray *et al.* 1993).

Metabolic activation of carcinogens by the cytochrome P450 system is a complex process with multiple enzymes involved in the generation of reactive intermediates as well as metabolites that are considered detoxification products. In addition, the expression and activity of specific P450 may be affected by multiple factors. They include polymorphisms in the structural gene itself, in receptors for inducers or other genes involved in controlling expression and by variable exposure to inducers, both exogenous xenobiotics and endogenous compounds. Similar variations in phase II enzymes, such as the glutathione *S*-transferases, will influence DNA adduct levels as will genetic or environmental factors influencing DNA repair capacity. Finally, cell turnover, particularly important in liver tissue which can regenerate, will also play a role. The studies presented here just begin to address this complex issue in humans.

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