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Immunohistochemical Study of CYP2E1 in Hepatocellular Carcinoma Carcinogenesis: Examination with Newly Prepared Anti-human CYP2E1 Antibody

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Abstract

Cytochrome P450 2E1 (CYP2E1) is known as a heme-containing enzyme that produces abundant free radicals, and its involvement in carcinogenesis has been suggested in several organs in vivo. In this study, to clarify the involvement of CYP2E1 in liver cancer and its carcinogenesis process, we investigated the expression of CYP2E1 in 42 surgically resected or biopsied specimens of hepatocellular carcinomas (HCC) and 26 cases with other liver lesions immunohistochemically using a newly prepared anti-human CYP2E1 antibody. When intracellular CYP2E1 expression was investigated in three different regions of HCC specimens, the expression in hepatocytes of the peri-tumor region was the highest ($p<0.001$) compared with those in the tumor and non-peri-tumor regions. Histologically, the expression of CYP2E1 in tumor cells tended to decrease as the cells were less differentiated ($p<0.0001$) and was the lowest in poorly differentiated HCC ($p<0.01$). CYP2E1 expression was highest in the pseudo-glandular type and low in the thick trabecular and solid types of HCC ($p<0.0001$). In mature regenerative nodules of liver cirrhosis, adenomatous hyperplasia (AH) and atypical adenomatous hyperplasia (AAH) to early-HCC, CYP2E1 expression was notably high as compared with other legions. CYP2E1 has a strong free radical-producing ability, and the cell injury and DNA damages by the free radicals are considered to be involved in carcinogenesis. Therefore, our results suggest that the different expression of CYP2E1 in hepatocytes may play important roles in the multistep carcinogenic process and the histogenesis of hepatocellular carcinoma.
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Key words: cytochrome 2E1, immunohistochemical expression, western blotting, carcinogenesis, hepatocellular carcinoma

Introduction

The multistep carcinogenesis process of hepatocellular carcinoma (HCC) has been clarified in recent years, in which cells progress from liver cirrhotic node (LC) to adenomatous hyperplasia (AH), atypical adenomatous hyperplasia (AAH) to early-HCC and HCC[1–3]. However, the mechanism of carcinogenesis in this process has not yet been clarified. Cytochrome P450 2E1 (CYP2E1) is an approximately 50 kD protein and a heme-containing enzyme which

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catalyzes the oxidation and reduction of a wide variety of endogeneous and exogenous substrates such as steroids, drugs and toxicants. It is mainly located in the membranes of endoplasmic reticulum and mitochondria and free microsomes, and is known to be induced in the liver by ethanol, isoniazid and other chemicals[4-6]. In addition to involvement in ethanol metabolism via the microsomal ethanol metabolic system (MEO) in long-term alcohol ingestion[7-8], CYP2E1 is involved in the metabolism of hepatotoxic substances such as carbon tetrachloride, and acetoaminophen and carcinogens such as N-nitrosodimethylamine[9-11]. CYP2E1 is also known to be a high producer of free radicals in vivo, suggesting its involvement in carcinogenesis [12-14]. Therefore, this study focused on CYP2E1 expression to clarify its involvement in the carcinogenesis process and histogenesis of HCC. We immunohistochemically investigated the CYP2E1 expression in surgically resected or biopsied HCC and related liver diseases using a newly prepared anti-human CYP2E1 antibody.

Materials and Methods

Materials

This study was performed using liver specimens resected at the First Department of Surgery of Nippon Medical School between June 1992 and February 1998. Forty-two cases with hepatocellular carcinoma (HCC), two with cholangiocarcinoma, 14 with liver metastasis of malignant tumors from other organs, two with adenomatous hyperplasia (AH), and three with benign liver tumors (two with focal nodular hyperplasias and one adenoma) who had not received preoperative therapy for liver lesions were investigated for this study. In addition, specimens from five LC patients biopsied or exploratorily resected as liver biopsy during laparotomy were examined as controls.

Among 42 HCC patients, those who underwent resection of one subregion or more and showed AH and AAH to early-HCC were included in both disease groups. After surgery, the resected specimens were fixed in 10% formaldehyde, embedded in paraffin, and sectioned at 3 μm thickness. These sections were stained with hematoxylin-eosin and observed under a light microscope. All samples were classified according to the General Rules for Clinical and Pathological Recording of Liver Cancer issued by the Japanese Liver Cancer Society[15]. The 42 cases with HCC were classified as well differentiated, moderately differentiated and poorly differentiated types and their histological types were classified as pseudo-glandular type, trabecular and solid type.

Table 1 Amino acid sequences of CYP 2E1 antigens

CYP49	ELKNIPKSFTRLAQ
CYP139	GMGKQGNESRIQRE
CYP402	EFPDPEKFKPEHFL

CYP number indicates a number from N-terminal of p450 amino acid sequence

For tumorous lesions in a subregion or wider liver resection, three regions were set for comparison:tumor cells (tumor region), peritumor hepatocytes within an area at 3 mm from the tumor margin (peritumor region), and hepatocytes in the non-peritumor region (non-peritumor region). In addition, the expression of CYP2E1 in hepatocytes in the non-peritumor region was compared with that in areas of chronic hepatitis activity (A_0: none to minimal, A_1: mild, A_2: moderate and A_3: severe) and fibrosis (F_0: no fibrosis, F_1: portal fibrous widening, F_2: portal fibrous widening with bridging fibrosis, F_3: bridging fibrosis plus lobular distortion and F_4: liver cirrhosis) in the background liver as assessed using the New Inuyama Classification established in 1995 [16].

For western blotting, the specimens from two livers without tumors and LC, two with LC and two with HCC were frozen and stored at −80°C without fixation.

All patients received an explanation of this experiment and gave informed consent to participate in the study.

Antibody

In rabbit polyclonal anti-human CYP2E1 antibody preparation by Nichirei (Nichirei, Co, Tokyo, Japan), the amino acid sequences of human CYP2E1 were analyzed. Three types of extremely hydrophilic regions were selected from 8 possible antigenic regions based on a previous report[17] and an antigenic index profile using a GENETYX software (Software Development Co., Ltd. Tokyo, Japan). The synthetic peptides for these regions were prepared by peptide synthesizer (**Table 1**). Briefly, rabbits were

immunized with each antigenic peptide conjugated with carrier protein BSA. IgG fraction was purified from rabbit antiserum using protein A column purification. After purification, rabbit polyclonal antibody was used as a primary antibody for human CYP2E1.

CYP2E1 402 selected based on the high titer represents the antigen peptide consisting of an amino acid sequence starting from the amino acid at position 402 from the N-terminal.

Western blotting method

The samples of normal liver, liver cirrhosis and hepatocellular carcinoma were homogenized in 5 ml glass tubes (Wheaton, Wheaton Science Product, NJ, USA) with buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% NP−40, 0.1% SDS, 1% DOC, 10 μg/m*l* leupeptin, 10 μg/m*l* aprotinin, 0.2 mM pefabloc SC, 1 uμ/ml pepstatin A, 1 mM sodium vanadate, and 20 mM sodium fluoride. Cell debris was removed by centrifugation at 20,000×g for 20 min at 4℃, and the supenatant was stored in aliquots at−80℃. The protein concentration of liver extracts was determined using the Brandford method[18]. Equal amounts of liver extracts (5 μg/Lane) were separated by 10% SDS-polyacrylamide gel electropholesis and were transferred to a polyvinyldifluoride membrane (Immobilone; Millipore, Bedford, MA, USA). The immunoblots of anti-human CYP2E1 402 antibody (dilution, 1: 20000, Nichirei, Tokyo, Japan) were detected with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) after the incubation of the alkaline phosphatase-conjugated second antibody.

Immunohistochemical Staining

The thin sections of each region set as described above were immunohistochemically stained by the streptavidin-biotin complex method (sABC kit, Nichirei, Tokyo, Japan) using anti-human CYP2E1 402 antibody (dilution, 1: 800, Nichirei, Tokyo, Japan) and observed under a light microscope. Staining of each cell was compared to that of bile duct epithelial cells near hepatocytes, and the staining intensity was divided into four groups in comparison with bile duct-ductule epithelial cell staining: negative, mildly positive, moderately positive, and markedky positive (**Fig. 1**). In addition, rank variables from 0 to 3 were set. Staining of CYP2E1 was investigated in each cell, and the staining grade that comprised the most dominant positive area was judged as the CYP 2E1 staining intensity in the cell group. The specificity of primary antibody was confirmed by absorption test using a synthetic peptide for human CYP2E1 (**Fig. 2**).

Statistical Analysis

For statistical analysis, Kruskal-Wallis rank test, Mann-Whitney's U test, and Spearman rank correlation were used, and a p value less than 0.01 was regarded as significant.

Results

Western Blotting

Western blotting using the anti-human CYP2E1 402 antibody showed that CYP2E1 protein was effectively expressed in all liver samples. In normal liver, liver cirrhosis and HCC, two bands were detected at approximately 51 kD and 54 kD (**Fig. 3**). Moreover, CYP2E1 in HCC (lane 5) showed a strong band at 51 kD similar to those in normal liver (Lane 1 and 2) and liver cirrhosis (Lane 3 and 4). In contrast, CYP2E1 in HCC (Lane 6) showed an intense band at approximately 54 kD.

Immunohistochemistry

Analysis of Histological Sites (Non-Tumor and Tumor Region) in Tumor Tissue

In patients with HCC who underwent surgical resection, intracellular CYP2E1 was immunohistochemically investigated in the tumor, peritumor, and non-peritumor regions. In hepatocytes in the non-peritumor region, the CYP2E1 staining intensity was almost the same as that in the nearby bile duct epithelial cells. In contrast, the intensity was strong in many hepatocytes in the peri-tumor region (**Fig. 4**). There were significant differences in the intracellular CYP2E1 staining intensity among the three regions ($p<0.0001$). The staining intensity in hepatocytes in the peri-tumor region was significantly stronger than in the tumor cells and hepatocytes in the non-peritumor cells ($p<0.001$, **Table 2**).

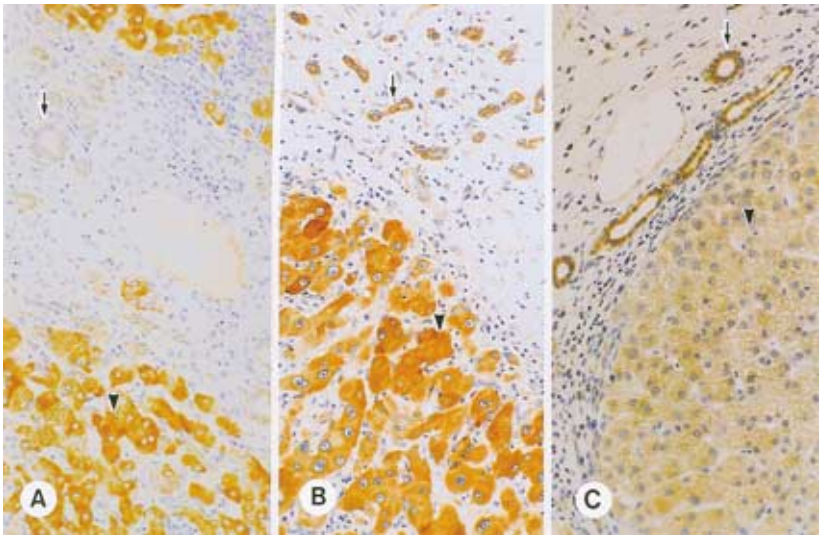

Fig. 1 The CYP 2 E 1 staining intensity of hepatocytes (arrowhead) was divided into four groups in comparison with bile duct-ductule epithelial cells (arrow): markedly positive (A), moderately positive (B), mildly positive (C) and negative staining (sABC method, ×50).

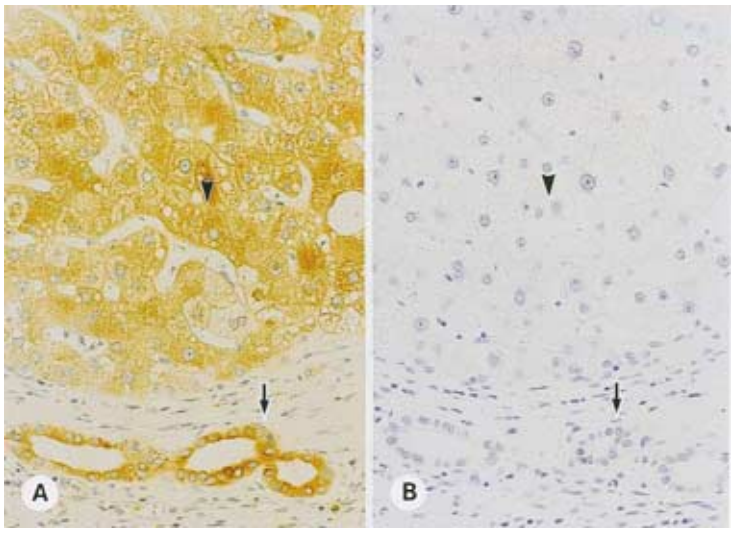

Fig. 2 A: The expression of CYP2E1 was observed in hepatocytes (arrowhead) and bile duct-ductule epithelial cells (arrow) (sABC method, ×80).
B: The expression of CYP2E1 in hepatocytes (arrowhead) and bile duct-ductule epithelial cells (arrow) was completely blocked with an antigen (sABC method, ×80).

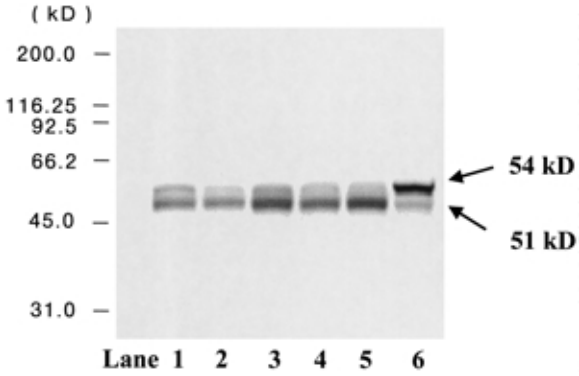

Fig. 3 Western Blotting for CYP2E1 in the Liver
In an immunoblot analysis of CYP2E1, 54 and 51 kD bands were observed in normal liver (Lane 1 and 2), liver cirrosis (Lane 3 and 4) and hepatocellular carcinoma (Lane 5 and 6)

Table 2 Expression of CYP2E1 according to the histological sites in tumor tissues

Site	No. of Cases	CYP2E1 Expression			
		Negative	Mild	Moderate	Marked
Tumor	55	1	16	20	18 *, *
Peri-Tumor	58	0	1	26	31 *
Non-Tumor	41	0	0	40	1 *

Kruskal-Wallis rank test $P<0.0001$ Mann-Whitney's U test *$P<0.001$
The staining intensity of hepatocytes in the peri-tumor region was significantly stronger than in the tumor cells and the non-peritumor cells ($p<0.001$).

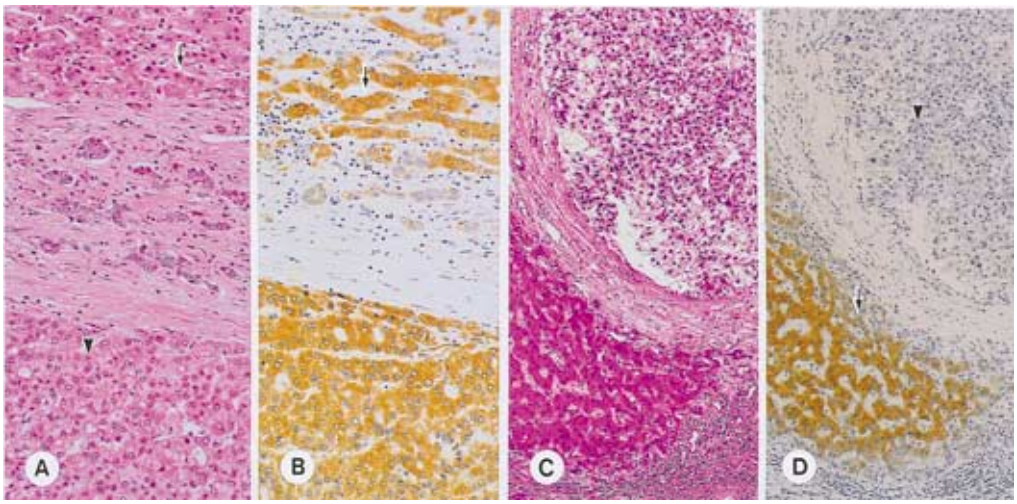

Fig. 4 The CYP2E1 staining intensity was significantly weaker in poorly differentiated HCC (D) than in well and moderately differentiated HCC (B). In contrast, the intensity was strong in many hepatocytes (arrow) in the peri-tumor region (arrowhead) (sABC method, ×33).
A and C: HE staining of same lesions (HE staining; ×33).

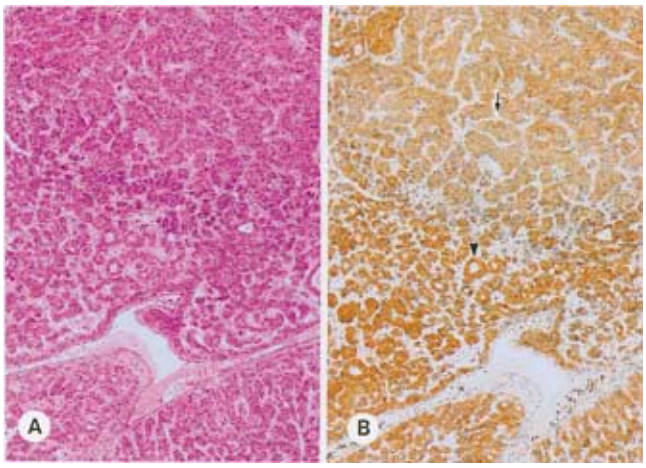

Fig. 5 A: The pseudo-glandular type HCC (arrowhead) and the trabecular and solid type HCC (arrow) (HE staining; ×33). B: The CYP2 E1 staining was strongest in the pseudo-glandular type HCC (arrowhead) compared to the trabecular and solid type HCC (arrow) (sABC method, ×33).

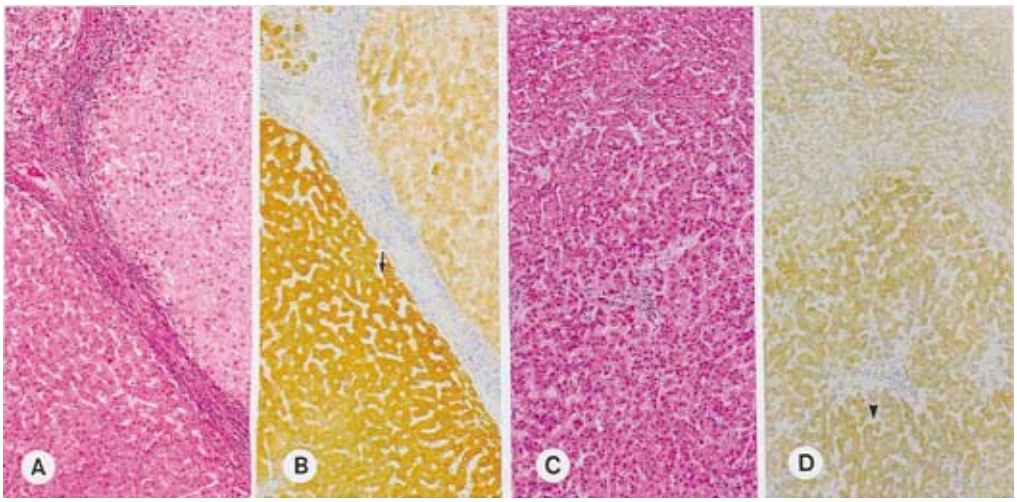

Fig. 6 The expression of CYP2E1 in (B): adenomatous hyperplasia (AH) (arrow), and (D): atypical adenomatous hyperplasia to early hepatocellular carcinoma (e-HCC) (arrowhead) was strongly stained (sABC method, ×33).
A and C: HE staining of same lesions (HE staining; ×33).

Table 3 Expression of CYP2E1 according to the histological grades of HCCs

Grade	No. of Cases	CYP2E1 Expression			
		Negative	Mild	Moderate	Marked
Well diff.	18	0	1	7	10
Moderately diff.	23	0	6	9	8
Poorly diff.	14	1	9	4	0

Kruskal-Wallis rank test $P<0.0001$

Mann-Whitney's U test *$P<0.01$, **$P<0.0001$

The staining intensity was significantly weaker in poorly differentiated HCC than in well and moderately differentiated HCC ($p<0.01$)

Table 4 Expression of CYP2E1 according to the histological types HCCs

Type	No. of Cases	CYP2E1 Expression			
		Negative	Mild	Moderate	Marked
Thin trabecular	15	0	1	7	7
Pseudo-glandular	10	0	0	0	10
Thick trabecular	21	0	10	9	2
Solid	9	1	5	3	0

Kruskal-Wallis rank test $P<0.0001$

Mann-Whitney's U test *$P<0.01$, **$P<0.001$

The staining was strongest in the pseudo-glandular type HCC ($p<0.05$), the next strongest staining was observed in the thin trabecular type ($p<0.05$), and the staining was weak in the thick trabecular and solid types.

Table 5 Expression of CYP2E1 according to the degree of chronic hepatitis activity in the background liver

Activity	No. of Cases	CYP2E1 Expression			
		Negative	Mild	Moderate	Marked
A_0	12	0	0	12	0
A_1	8	0	0	6	2
A_2	10	0	0	10	0
A_3	39	0	1	37	1

Spearman rank correlation $p=0.3041$

The degrees of chronic hepatitis activity did not affect the CYP2E1 staining in hepatocytes.

Table 6 Expression of CYP2E1 according to the degree of fibrosis in the background liver

Fibrosis	No. of Cases	CYP2E1 Expression			
		Negative	Mild	Moderate	Marked
F_0	20	0	0	19	1
F_1	2	0	0	2	0
F_2	7	0	0	6	1
F_3	11	0	0	11	0
F_4	29	0	1	27	1

Spearman rank correlation $p=0.3792$

The degrees of fibrosis did not affect the CYP2E1 staining in hepatocytes.

Analysis of Histological Grade

Staining intensity in the tumor cells was compared among well, moderately, and poorly differentiated types of HCC (**Fig. 4**), and there were significant differences in the staining intensity among the three types ($p<0.0001$). The staining intensity in the tumor cells tended to decrease as the histological grade was less differentiated, and the staining intensity was significantly weaker in poorly differentiated HCC than in well and moderately differentiated HCC ($p<0.01$, **Table 3**).

Analysis of Histological Type

HCC formed thin trabecular, thick trabecular, pseudo-glandular and solid type structures, and the staining in the tumor cells was compared among these structure types. There were significant differences in the staining intensity among the four types ($p<0.0001$, **Fig. 5**). The staining was strongest in the pseudo-glandular type HCC ($p<0.05$), the next strongest staining was observed in the thin trabecular type ($p<0.05$), and the staining was weak in the thick trabecular and solid types (**Table 4**). In the cancer cells forming both pseudo-glandular and trabecular type structures in the same tumor, the CYP 2E1 staining was clearly stronger in the tumor cells forming the pseudo-glandular structure.

Analysis of Histological Activity and Fibrosis in the Background Liver

Chronic hepatitis activity and fibrosis in the background liver were divided into four and five grades, respectively, and their correlations with the staining of hepatocytes in the non-peritumor region were investigated. In this study, the degrees of chronic hepatitis activity (**Table 5**) and fibrosis (**Table 6**) did not affect the staining in hepatocytes.

Staining of Pre-cancerous and Other lesions

Although the number of patients was small, the staining was notably stronger in mature regenerative nodules of liver cirrhosis, AH, and AAH to early-HCC compared to that in the nearby cells (**Fig. 6**). In surgically resected non-hepatocellular liver tumors such as cholangiocarcinoma, metastatic liver cancers and benign liver tumors, intracellular CYP2E1 was also immunohistochemically detected in the tumor, peritumor and non-peritumor regions. However, there were no significant differences among the three regions in any tumor.

Discussion

The theory of characteristic multistep carcinogenesis of HCC has been generally accepted in recent years, and AH has been attracting attention as a precancerous lesion[1-3]. However, in spite of various grounds, a constant overlook has not yet been established for the mechanism of a stepwise carcinogenesis process showing how AH occurs and how AH progresses to HCC. CYP2E1 has a strong free radical-producing ability due to the metabolism of drugs and chemicals[4-11], and this free radical causes cytotoxicity such as cell membrane and protein injuries, suggesting that CYP2E1 is secondarily involved in carcinogenesis[13-14]. In this study, we investigated the role of CYP2E1 in the multistep carcinogenesis of HCC by immunohistochemical staining of resected specimens including biopsy specimens with a newly prepared anti-human CYP2E1 antibody. In western blotting of normal liver, LC and HCC, two bands for CYP2E1 were detected at approximately 51 kD and 54 kD in all liver samples. It has been reported that in human tissues, lymphocyte microsomes expressed 51 kD form and liver microsomes 54 kD and 51 kD forms of CYP2E1 due to either variations in the amount of microsomal protein or structural alterations[19]. The 54 kD membrane-binding form and 51 kD cytosol form of CYP2E1 in hepatocytes have been also reported in mammalians[20]. Variations in the expression and structural differences of CYP2E1 were ascribed to pathophysiological conditions such as diabetes mellitus and exposure of xenobiotics[21]. In this study, different patterns of the two bands for CYP2E1 were observed in HCC tissues. One pattern existed in histologically well to moderately differentiated type as a strong band at 51 kD, similar to those in normal liver and LC.

The other pattern was found in poorly differentiated types as an intense band at 54 kD (**Fig. 3**). Although further investigations are necessary, these findings suggest a relationship between the two

paterns of CYP2E1 expression in HCC and histological differentiation of the tumors. Furtheremore, these alteration of bands and structural alterations may also play an important role in histogenesis and carcinogenesis of HCC.

Immunohistochemically, we confirmed strong intracellular CYP2E1 expression in mature liver cirrhotic nodes and LC. Although the mechanism by which CYP2E1 was induced in the cells is still unknown, it may be suggested that these high-risk cells or precancerous lesions could be cancerized by cell injury and DNA damages caused by free-radical produced by CYP2E1. Although mature liver cirrhotic nodes and AH could not be assessed due to the small number of patients, strong CYP2E1 expression was observed in the peritumor region located within 3 mm of the tumor margin in patients with HCC. This finding suggested that in addition to the well-known induction by ethanol and isoniazid, certain chemical mediators and cytokines[22-24] released from hepatocytes might also induce CYP2E1 in cells in the peritumor region. Therefore, CYP2E1 is considered to be involved in the multistep carcinogenesis of HCC. In this process the background liver may be changed from chronic hepatitis to liver cirrhosis by persistent inflammatory reactions, and CYP2E1 may be somehow induced in the cytoplasm of hepatocytes in cirrhotic nodes as a maturity feature. In addition, free radicals produced by CYP2E1 may induce damages to proteins and DNA. These cellular damages may cause progression of mature liver cirrhotic nodes to AH and HCC. Tumor emboli in portal veins and cancerous lesions that proliferated from tumor emboli have been diagnosed as intrahepatic metastasis[25]. Many small cancerous lesions close to the main cancerous lesion, gradually decreasing lesions far from that, and cancerous lesions showing the same histological type or lower histological grade than the main lesion have been diagnosed as intrahepatic metastasis, too[26]. Actually, some cancerous lesions are difficult to determine as primary or metastatic. Our study suggested that cytokines released from a HCC and certain chemical mediators might induce CYP2E1 in the peritumor region, implying that free radicals produced by CYP 2E1 in these cells cause severe cell injury, probably leading to the above-mentioned cancerous lesions of previously unknown causes.

Although further investigation and discussion would be necessary to determine the relationship between CYP2E1 expression in tumor cells and the histological grade and type of HCC, we found that the CYP2E1 expression in the tumor cells was increased as the tumors were more differentiated type. It was considered that well differentiated HCC cells were more maturated than poorly differentiated HCC cells, and mature cancer cells had sufficient CYP2E1-inducing function. Regarding the histological type, strong CYP2E1 expression was observed in the tumor cells of pseudo-glandular type and thin trabecular type. Patients with this histological type in our series had relatively differentiated cancer cells, which may be one of the causes of high CYP2E1 expression, but it is very interesting that the CYP2E1 expression was particularly high in tumor cells forming a pseudo-glandular structure.

The major inflammatory lesion in chronic hepatitis appears in the area containing the portal vein, and prolonged inflammation in this area involves hepatocytes in the hepatic marginal lobular region, resulting in histological changes such as piecemeal necrosis. Then, hepatocyte injury and subsequent fibrous outgrowth are expanded, followed by the appearance of regenerated nodes, leading to LC. Although the degrees of inflammatory activity and fibrosis vary, hepatocytes may be injured by persistent inflammation during this process. However, in this study, there were no significant differences in the CYP2E1 expression in hepatocytes among the different degrees of inflammatory activity and fibrosis, suggesting that these factors do not affect the CYP2 E1 expression. Accordingly, it was concluded that CYP2E1 may be induced by certain factors upon maturation of liver cirrhotic nodes, which is very interesting. In the future, it is necessary to investigate CYP2E1 inducers that may appear in hepatocytes during embryogenesis, maturation of liver cirrhotic nodes and carcinogenesis, though several genetic mechanisms involving genetic polymorphisms of CYP2E1 have been reported[27-29].

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