# Protein level of apolipoprotein E increased in human hepatocellular carcinoma

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Received October 3, 2005; Accepted November 28, 2005

**Abstract.** Many reports suggest that hepatic steatosis leads to hepatocellular carcinoma (HCC), including hepatitis C virus or non-alcoholic steatohepatitis. Proteomic study of tumor tissues from HCC patients, focusing on apolipoprotein (apo) of apoA1, apoB100 and apoE, was performed by immunoblotting. Although the significant changes of apoA1 or apoB100 could not be shown statistically, the immunoblotting showed the increase in protein level of apoE in the tumor tissues of 88% of patients without increase of apoE gene expression and serum level. These results suggest the accumulation of apoE by impaired secretion. Moreover, immunoblot analysis on two-dimensional electrophoresis showed a strong possibility that sialylated forms of apoE also were increased in tumorous tissues of HCC. ApoE level in tumorous tissues is frequently elevated and may be a good histological marker for HCC.

## Introduction

Chronic infection with hepatitis B virus (HBV) or C virus (HCV) can cause hepatocellular carcinoma (HCC). Most Japanese patients with HCC are also infected with HCV, indicating that this virus plays an important role in hepatocarcinogenesis. Specific markers for HCC, if present, are reliable for early detection and for gaining an improved understanding of hepatocarcinogenesis.

α-fetoprotein and protein induced by vitamin K absence-2 (PIVKA-2) are clinically applied as tumor markers of HCC. However, the sensitivity of these markers is low for early HCC. Therefore, many proteomic studies of HCC have investigated the levels of several proteins in tissues from patients with HCC (1-8). We found that the expression of heat shock protein 70 family members increases, whereas that of aldolase or arginase 1 decreases in HCV-related HCC tissues (6,7). Proteomic analysis should identify new tumor markers. We found the decrease of mitochondrial enoyl-CoA hydratase in HCV-related HCC tissues by proteomic analysis (7). The decrease of enoly-CoA hydratase leads to damage of ß-oxidation and lipid metabolism. A relationship between HCC and lipid metabolism has been argued and many studies have found that hepatic steatosis leads to HCC, especially when HCV is involved (9-11). Furthermore, HCV is closely associated with the metabolism of lipids, especially lipoproteins (12-16). Low-density lipoprotein (LDL) receptor might help transport HCV into hepatocytes (14). The inhibition of microsomal triglyceride transfer protein (MTP) activity by HCV core proteins causes hepatic steatosis due to the reduced secretion of very low-density lipoprotein (VLDL) from hepatocytes (17,18). It has been reported that apolipoprotein (apo) E works as a ligand of LDL receptor (19) and apoE is a component of VLDL. Accordingly, we supposed that the accumulation of VLDL or apoE may cause hepatic steatosis and consequently lead to hepatocarcinogenesis. Moreover, some reports recently showed that apoE expression increases in the malignancy such as prostate (20), gastric (21) and ovarian cancers (22). Therefore, we examined the relationship between HCC and apoE.

## Materials and methods

*Tissue specimens*. We examined tumorous and paired non-tumorous liver specimens from 17 patients who had undergone partial hepatectomy for HCC at Yamaguchi University Hospital between 1998 and 2000.

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*Abbreviations*: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; apo, apolipoprotein; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MTP, microsomal triglyceride transfer protein; HCV-Ab, anti-hepatitis C virus antibody; HBs-Ag, hepatitis B surface antigen; PIVKA-2, protein induced by vitamin K absence-2; 2ME, 2-mercaptoethanol

*Key words*: apolipoprotein E, hepatocellular carcinoma, O-linked glycoprotein

Table I. Clinical backgrounds on patients with HCC.

| Pts. | Age | Sex | HCV-Ab | HBs-Ag | Child-Pugh grade (21) | Sera total cholesterol level (130-220 mg/dl)[a] | History of therapy for hyperlipidemia | Drinking habits | Complications of diabetes mellitus |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 75 | Female | (+) | (-) | A | [b] | (-) | [b] | [b] |
| 2 | 60 | Male | (+) | (-) | A | 162 | (-) | (+) | (+) |
| 3 | 74 | Female | (+) | (-) | A | 159 | (-) | (-) | (-) |
| 4 | 64 | Male | (+) | (-) | A | 189 | (-) | (-) | (-) |
| 5 | 50 | Male | (+) | (-) | A | 143 | (-) | (+) | (-) |
| 6 | 68 | Male | (+) | (-) | A | 174 | (-) | (-) | (-) |
| 7 | 65 | Female | (+) | (-) | A | 192 | (-) | (-) | (-) |
| 8 | 58 | Male | (+) | (-) | A | 166 | (-) | (-) | (-) |
| 9 | 66 | Male | (+) | (-) | A | 152 | (-) | (-) | (-) |
| 10 | 76 | Female | (+) | (-) | A | 171 | (-) | (-) | (-) |
| 11 | 73 | Female | (+) | (-) | A | 139 | (-) | (-) | (+) |
| 12 | 65 | Male | (+) | (-) | A | 112 | (-) | (+) | (-) |
| 13 | 69 | Male | (+) | (-) | A | 156 | (-) | (+) | (-) |
| 14 | 58 | Male | (+) | (-) | A | 178 | (-) | (+) | (+) |
| 15 | 57 | Male | (-) | (+) | A | 176 | (-) | (-) | (-) |
| 16 | 30 | Female | (-) | (+) | A | 163 | (-) | (-) | (+) |
| 17 | 59 | Male | (-) | (+) | A | 150 | (-) | (+) | (-) |

[a]The cholesterol level was the values at the admission to our hospital. [b]Not indicated in the medical sheet.

Table I shows the clinical background of patients bearing HCC. Fourteen were positive for anti-HCV antibody (HCV-Ab) and 3 were positive for hepatitis B surface antigen (HBs-Ag). The liver function of all patients was Child-Pugh grade A (23). Although 6 of the 17 patients had an alcohol habit and 4 had diabetes mellitus, none had undergone treatment for hyperlipidemia before the hepatectomy, and the total serum cholesterol levels in the 16 patients were normal at the time of admission to Yamaguchi University Hospital. Table II shows the clinical tumor data. The TNM staging was based on the criteria of the Liver Cancer Study Group of Japan (24). The HCC tumors from 2, 13 and 2 patients were well, moderately and poorly differentiated, respectively. Cirrhosis of the liver was indicated in 9 of the 17 non-tumorous tissues. We also included normal liver tissues from 8 patients who had undergone partial hepatectomy for metastatic liver tumors. We obtained serum samples from 3, 8 and 5 patients with HBV-related HCC, HCV-related HCC and metastatic liver tumors, respectively.

*Sample preparation*. Resected liver tissues that had been immediately frozen in liquid nitrogen and stored at -80˚C were disrupted in lysis buffer (1% NP-40, 1 mM vanadic acid, 1 mM PMSF, 50 mM Tris, 10 mM NaF, 10 mM EDTA, 165 mM NaCl, 10 $\mu$g/ml leupeptin and 10 $\mu$g/ml aprotinin) using a Potter type homogenizer with a Teflon tip at 4˚C for 1 h. The homogenate was separated by centrifugation at 15,000 x g for 30 min to yield a supernatant that was stored at -80˚C.

Morning blood samples collected from patients who had fasted overnight were allowed to clot at room temperature. After centrifugation at 3,000 rpm for 15 min, the serum was decanted and stored at -80˚C.

Table II. Clinical tumor data in HCC.

| Pts. | Main tumor size (cm) | TNM stage[a] | Differentiation |
|---|---|---|---|
| 1 | 2.8 | II | Moderately |
| 2 | 5 | II | Moderately |
| 3 | 2.9 | I | Moderately |
| 4 | 3.4 | II | Poorly |
| 5 | 3.5 | II | Moderately |
| 6 | 3.8 | I | Moderately |
| 7 | 2 | II | Moderately |
| 8 | 2.5 | I | Well |
| 9 | 2.5 | II | Moderately |
| 10 | 9 | II | Moderately |
| 11 | 1.2 | I | Well |
| 12 | 13 | III | Moderately |
| 13 | 7 | III | Moderately |
| 14 | 12 | IV | Moderately |
| 15 | 2.9 | II | Poorly |
| 16 | 12 | IV | Moderately |
| 17 | 3.5 | I | Moderately |

[a]TNM staging by the Liver Cancer Study Group of Japan criteria (24).

*Two-dimensional gel electrophoresis (2-DE)*. Protein samples (50 $\mu$g) each were applied to Immobiline dry strips (pH 3.0 to 10.0) 7 cm, Amersham Pharmacia Biotechnology, Uppsala,

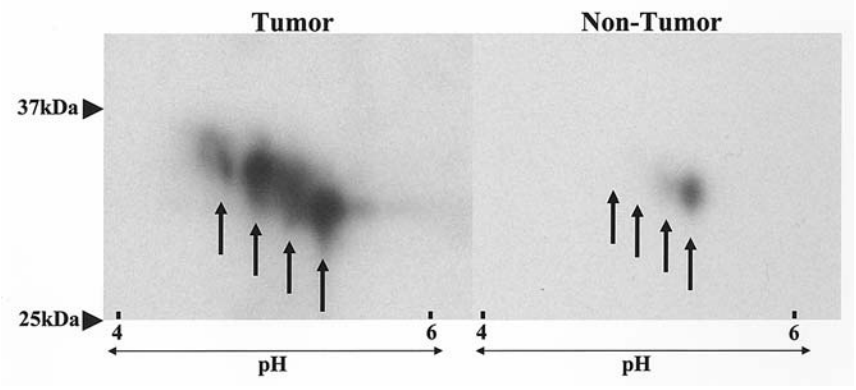



Figure 1. 2-D immunoblotting analysis with anti-apoE antibody in liver tissues. Immunoblotting analysis with anti-apoE antibody was performed after 2-DE. The protein levels of apoE in tumorous tissue were higher than in non-tumorous tissue.

Sweden) in a total volume of 125 $\mu$l containing 8 M urea, 2% CHAPS and 0.5% IPG buffer (Amersham Pharmacia Biotechnology) and 0.56% 2-mercaptoethanol (2ME). After rehydration for 14 h, proteins were separated by isoelectrofocusing (IEF) at 20˚C and 50 $\mu$A/strip with the following linear voltage increases: 500 V for 1 h, 1,000 V for 1 h and 8,000 V for 2 h. The strips were then equilibrated twice in 50 mM Tris containing 6 M urea, 30% glycerol and 2% SDS for 10 min. Then 2ME was added, followed by iodoacetamide. The second dimension proceeded on 12.5% non-gradient SDS-polyacrylamide gels at two steps: 600 V, 20 mA for 30 min and 600 V, 50 mA for 70 min in a Multiphor horizontal electrophoresis unit (Amersham Pharmacia Biotechnology).

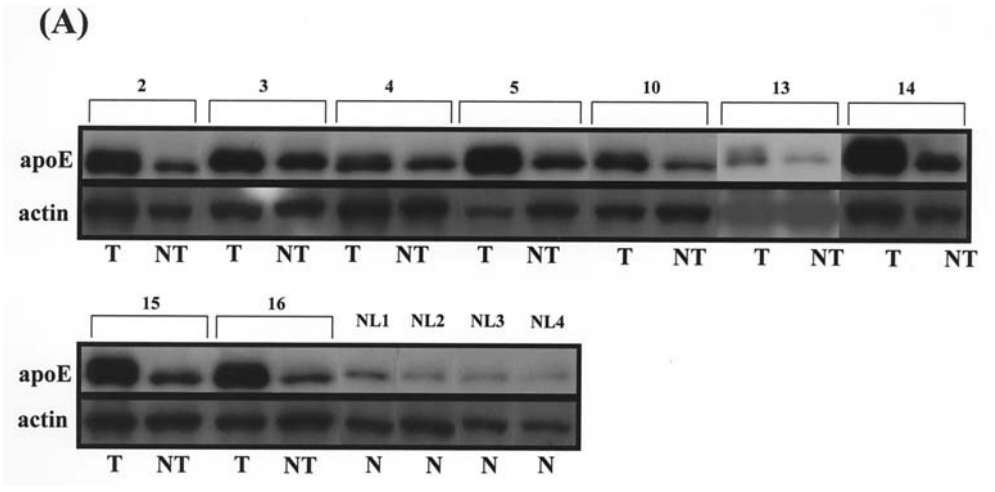
*Immunoblotting*. Liver and serum protein samples (20 and 10 $\mu$g, respectively) were resolved by SDS-PAGE at 15 mA/gel using 10-20% gradient polyacrylamide gel. After SDS-PAGE, fractionated proteins were transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membrane (Immobilon; Millipore Corporation, Bedford, MA) and blocked overnight at 4˚C with TBS containing 5% skim milk. The primary antibodies were HRP-conjugated goat anti-human apolipoprotein E antibody (1:2,000), apolipoprotein A1 (1:4,000), apolipoprotein B100 (1:2,500) (all from Academy Biomedical Company, Houston, TX) and rabbit anti-human actin antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were incubated for 1 h, washed four times with TBS containing 0.05% Tween-20 and visualized using a chemiluminescence reagent (ECL; Amersham Pharmacia Biotechnology).

*Image analysis*. The positions of the protein bands on the gels were defined using an Agfa ARCUS 1200™ image scanner (Agfa-Gevaert N.V., Mortsel, Belgium) and analyzed with Image Gauge ver. 3.45 software (Fuji Film Science Lab, Minami Ashigara, Japan). We applied the representatives of each gel on the same gel to do away with the difference between some gels, and made revisions in the density of apoE.

*Immunohistochemistry*. Paraffin-embedded blocks of liver samples from both groups and 3 $\mu$m sections were examined by immunohistochemistry using the standard avidin-biotin-peroxidase complex (ABC) method. Sections that were dewaxed in xylene and dehydrated in alcohol were heated in a microwave oven for 6 min to activate antigens and then endogenous peroxidase activity was blocked by immersing the sections for 30 min in 0.3% hydrogen peroxidase in methanol. The sections were washed with distilled water, incubated in PBS containing rabbit serum for 20 min to block non-specific binding and then incubated with anti-human apolipoprotein E antibody (goat, 1:50; Santa Cruz Biotechnology) overnight at 4˚C. The sections were incubated with biotin-conjugated secondary antibody in PBS for 60 min at room temperature, and reacted with ABC for 30 min. Positive reactions were developed for about 3 min using PBS containing hydrogen peroxidase and 3,3'-diaminobenzidine (DAB).

## Results

First we carried out the immunoblot analysis of apoE on the 2-DE map in both tumorous and non-tumorous tissue. ApoE was detected as four spots in tumorous tissue and three of the four spots had lower pI and higher molecular weight than the spot of apoE in non-tumorous tissue. The density of apoE spots in the tumorous tissue was obviously higher than in non-tumorous tissue (Fig. 1). Quantitative analysis of ApoE was performed by SDS-PAGE and immunoblotting of paired tumorous and non-tumorous liver tissues from 17 patients with HCC followed by image analysis. Protein levels of apoE in tumorous liver tissues were obviously increased in 13 of the patients compared with non-tumorous liver tissues, and slightly increased in 2 of them. The amounts of apoE expressed in non-tumorous liver tissues of HCC and normal liver tissues did not differ (Fig. 2A). ANOVA with Bonferroni-Dunn test revealed that the density of bands in tumorous liver tissues was significantly higher than that in non-tumorous liver tissues (Fig. 2B). However, the serum apoE levels from the patients with and without HCC did not differ (Fig. 3). Previously, Iizuka *et al* analyzed the mRNA expression of apoE in HCC tissues using cDNA microarrays and our samples and showed that apoE mRNA level does not change compared with normal liver tissues (25). We performed immunohistochemical studies to check the cells expressing apoE. The analysis showed apoE expression in the cancer cells and hepatocytes in non-tumorous tissues, and the stain in the tumorous tissues is stronger than in the non-tumorous tissues. Granular pattern of apoE was observed around the nuclei (Fig. 4).



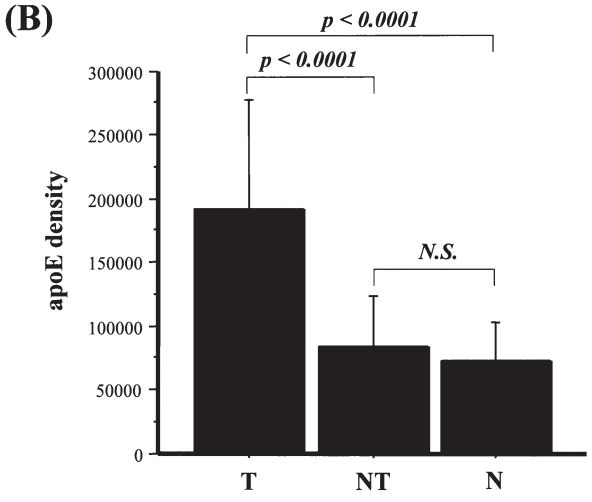




Figure 2. 1-D immunoblotting analysis with anti-apoE antibody in liver tissues. (A) Immunoblots of apoE and ß-actin. Samples are 9 of 17 HCC and 4 of 8 metastatic liver tumors. Levels of total apoE are higher in tumorous (T) than in non-tumorous (NT) and normal liver (N) tissues. Lanes 2-5, 10, 13 and 14 show 7 of 14 HCV-related HCCs; lanes 15 and 16 show 2 of 3 HBV-related HCCs; NL 1-4 show 4 of 8 metastatic liver tumors. Numbering of patients with HCC corresponds to that in Table I. NL, patients of metastatic liver tumors. (B) Comparison of apoE density in normal, tumorous and non-tumorous HCC tissues. Levels of apoE in tumorous tissues are significantly higher than those in non-tumorous and normal tissues. However, non-tumorous and normal tissues did not differ. This quantitative study was performed by ANOVA with Bonferroni-Dunn test. $p<0.0167$ was considered significant. The error bar shows mean ± SD. T, tumorous tissues of HCC; NT, non-tumorous tissues of HCC; N, normal liver tissues from the patients with metastatic liver tumor.

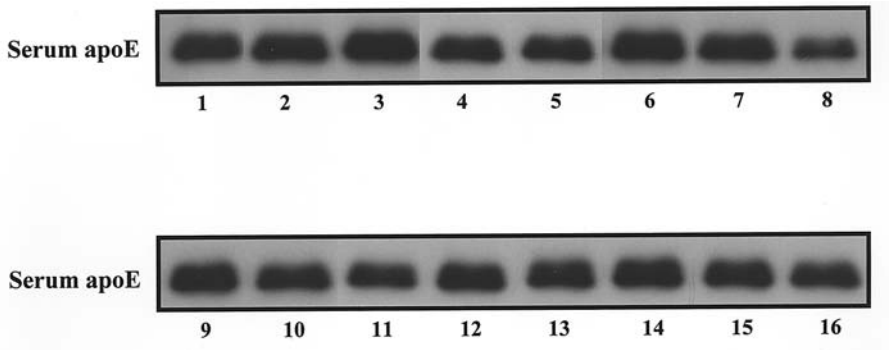



Figure 3. Immunoblotting analysis of apoE in sera from patients with HCC. Serum apoE levels were not increased in patients with HCC. Sera from patients with HCV-related HCCs (lanes 1-8); HBV-related HCCs (lanes 9-11) and metastatic liver tumors (lanes 12-16).

And we also investigated the protein level of apoA1 and apoB100 in HCC tissues. ApoA1 is a major component of HDL and apoB100 is a major component of LDL. The apoA1 and apoB100 level in HCC tissues hardly changed (data not shown).

## Discussion

Apolipoproteins A1, B100 and E in HCC were analyzed by 2-DE and immunoblotting. Only the protein level of apoE significantly increased in HCC tissues. ApoE levels did not correlate with size, stage or differentiation of HCC tissues (data not shown). ApoE is a glycoprotein with a molecular weight of 34 kDa and is a component of chylomicrons, VLDL and high-density lipoprotein (HDL). Apo E is a ligand for the LDL receptor or remnant receptor that is involved in lipid metabolism by transporting triglyceride-rich lipoproteins into cells (19). ApoE has three main isoforms E2, E3 and E4. Our results showed that 72.7% of HCC patients were E3 homozygotes of wild-type (data not shown). The human

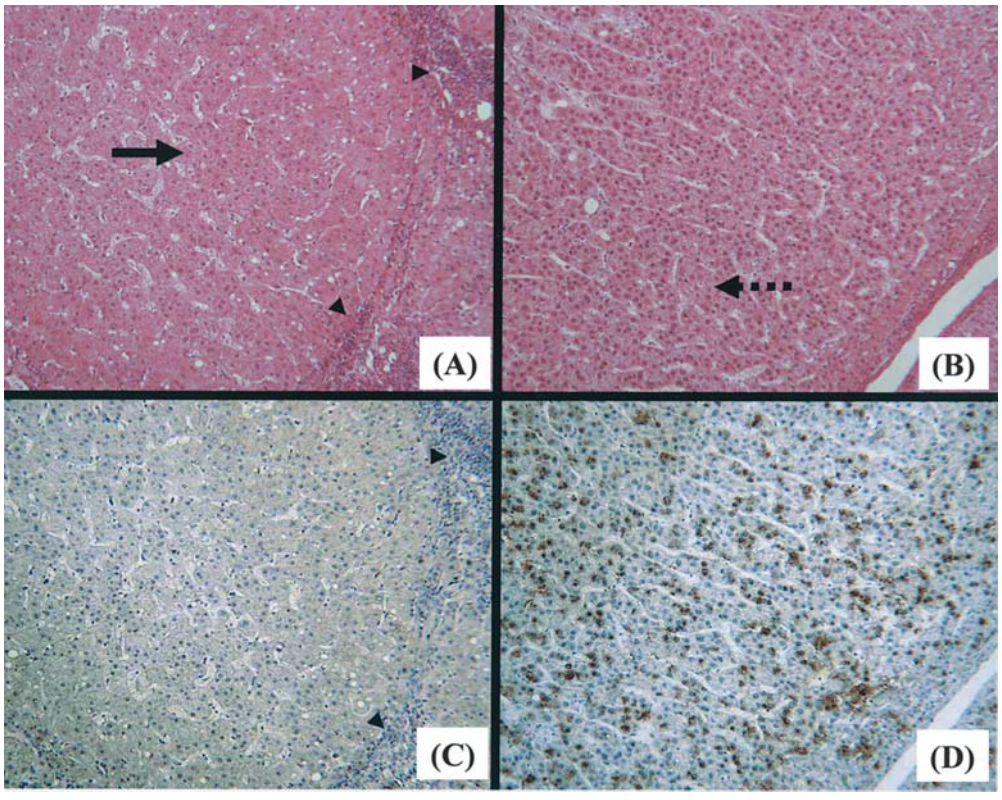



Figure 4. Immunohistochemical analysis of apoE in tumorous and non-tumorous tissues of HCC. Hematoxylin-eosin staining (A) non-tumorous lesion and (B) tumorous lesion in liver tissues of HCC patients. Magnification x200. Immunostaining of apoE in non-tumorous (C) and in tumorous tissues (D), magnification x200. (A) The solid arrow indicates non-tumor hepatocytes. The triangles show the cirrhotic change with inflammatory cells infiltration into Glisson's capsule. (B) The dotted arrow indicates hepatoma cells with acinar-like formation, and apoE is positively stained in the cells.

hepatoma cell line HepG2 (26) and fetal hepatocytes (27) secrete apoE. Our results indicated that the protein level of apoE is significantly increased in HCC tissues, although serum apoE levels did not differ between patients with and without HCC. This suggests that apoE secretion is reduced, or that apoE production is increased in HCC tissues. The core proteins of HCV inhibit MTP activity and VLDL secretion, which consequently leads to hepatic steatosis (17,18). Reduced VLDL secretion also causes hepatic steatosis in apoE knockout mice (28-30) and in the mice that are lacking normal apoE function (31). It has been reported that the inhibition of VLDL secretion by HCV core protein or a mutation of apoE causes apoE to accumulate in HCC tissues. ApoE is produced and secreted by human hepatoma cell lines (32,33) as well as by fetal liver (27), and the expression of apoE increases during embryonic development in zebrafish (34). Moreover, oxidative stress in HCC tissues (35) causes high levels of apoE expression because apoE functions as an anti-oxidant, and apoE mRNA expression increases in prostate cancers with high malignant potential (20), in gastric (21) and ovarian cancers (22). These reports support the latter notion. Iizuka *et al* (25) showed using cDNA microarrays and our samples that although the apoE mRNA level does not change, expression of the LDL receptor significantly decreases in HCC ($p=0.0063$) compared with normal liver tissues. Pan *et al* (36) reported that lipid peroxidation inhibited the secretion of apoE. These findings support the notion that the increased apoE protein level in HCC tissues is due to reduced apoE secretion without increased apoE intake into cancer cells. The difference of mRNA expression of apoE between gastric or ovarian cancer tissues and HCC cancer tissues may result from the difference of expression in non-tumorous tissues. ApoE is mainly produced by hepatocytes, macrophages and astrocytes but detectable in most peripheral tissues. Accordingly, mRNA of apoE in the normal liver is at a high level. Therefore, HCC cancer cells may keep the high protein level of apoE by reducing the secretion from cancer cells without increase the expression of apoE. Some reports revealed that apoE is connected with growth arrest or apoptosis (22,37). Chen *et al* demonstrated that the decrease of apoE expression leads to G2 cell cycle arrest and apoptosis in apoE-expressing ovarian cancer cell line using RNA*i* technique (22). These data supposed that the protein level of apoE in cancer cells may not increase but apoE itself may be related to the proliferation or survival in cancer cells. However, we could not directly demonstrate this hypothesis.

The immunoblotting against apoE on 2-DE showed that the four spots of apoE were detected in HCC tumorous tissues and all four spots in tumorous tissues were denser than in non-tumorous tissues (Fig. 1). It is post-translationally modified by O-linked glycosylation, including sialic acid at 194-threonine (38). A previous report demonstrated that the spots of sialylated apoE on 2-DE locates in the upper and acidic side of asialo-apoE (39). These findings indicate the possibility that not only total apoE level but also the level of its sialylated form may be elevated in human HCC tumorous tissues. Sialyltransferase is known as an enzyme for

sialylation. Sialyltrans-ferase activities are increased in several cancers (40-42) and either α 2,3-sialyltransferase or α 2,6-sialyltransferase catalyzes apoE sialylation. Iizuka *et al* (25) found using a cDNA microarray that the expression of several sialyltransferases did not significantly increase in a study of our samples, whereas that of ß-galactoside α 2,3-sialyltransferase tended to increase in HCC tissues (p=0.062). Furthermore, levels of α 2,6-sialylated glycoconjugates are significantly increased in HCC tissues (43). Thus, these enzymes might be activated in HCC tissues.

The protein levels of apoE evidently increased in human HCC tissues whereas the serum apoE level did not. This suggests that protein level of apoE is increased in human HCC tumorous tissues without increase of mRNA expression because of reduced secretion of apoE from cancer cells and that sialylation of apoE may be related to increase of apoE in HCC tissues.

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