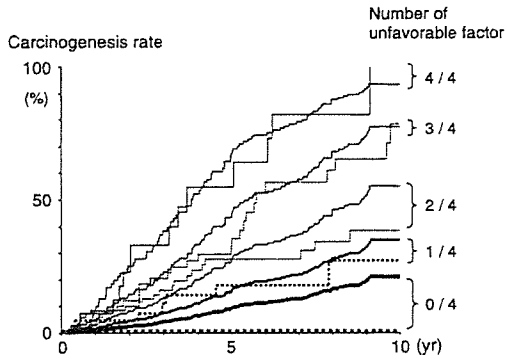

Fig. 3. Simulated HCC appearance curves with actual appearance rates of internal and external validation cohorts, according to the number of unfavorable risk factors. Five solid curves show simulated carcinogenesis rates drawn according to the number of unfavorable risk factors; none (the thickest line), one, two, three, and four (the thinnest line). Five dotted curves indicate actual HCC appearance curves of the validation cohort (Toranomon Hospital, 1991–2003).

development that combine several variables of patient data to indicate the probability of clinical outcome are powerful tools for assisting physicians in the decision-making process. Our model can be used for prediction of HCC in daily clinical practice by hepatologists, for education and information for individual patients, for selection of a candidate for a cancer prevention program, and for a proper stratification of cirrhotic patients in clinical trials for the purpose of cancer prevention. The consistency and reproducibility of the present model should also be confirmed by other institutions outside Japan.

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HEPATOLOGY

Origin of neovascular structure in an early stage of hepatocellular carcinoma: Study of alpha-smooth muscle actin immunohistochemistry in serial thin sections of surgically resected cancer

KENJI IKEDA, MASAHIRO KOBAYASHI, SATOSHI SAITOH, TAKASHI SOMEYA, TETSUYA HOSAKA, HITOMI SEZAKI, YOSHIYUKI SUZUKI, FUMITAKA SUZUKI, NORIO AKUTA, YASUJI ARASE AND HIROMITSU KUMADA

Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan

Abstract

Background: To elucidate the origin of the neovascular structure found in well-differentiated hepatocellular carcinoma (HCC), an immunohistochemical study was performed on sequential thin section specimens.

Method: Eleven surgically resected specimens of well-differentiated HCC were analyzed for neovascular structure using monoclonal alpha-smooth muscle actin (α-SMA) antibody. Each paraffin specimen was serially sliced to a thickness of 3 μm for immunohistochemistry. When a ring-shaped structure was found unrelated to portal triads on α-SMA staining, it was regarded as abnormal neovascularity (non-triadal vessel or unaccompanied vessel).

Results: All of the 11 liver cancers had thin-walled, round- or oval-shaped non-triadal vessels in their well-differentiated parts. Immunohistochemistry of serial thin sections of HCC showed that these non-triadal vessels were connected to portal veins in portal triads in well-differentiated cancer in a total of nine patients (81.8%). This type of neovascular structure found in a well-differentiated cancer seemed to be a surviving portal vein among diminishing and disappearing arteries and bile ducts. All 11 tumors showed isovascular staining on ordinary digital subtraction angiography, and four of the tumors showed negative enhancement on intra-arterial carbon dioxide-enhanced ultrasonography or computerized tomographic (CT) hepatic arteriography, suggesting a relative arterial blood scarcity in the tumor nodules.

Conclusion: At an early stage of HCC, non-triadal vessels originate from ordinary portal veins in intratumoral portal triads. This fact sufficiently explains the reason why a well-differentiated liver cancer can sometimes show arterial blood paucity on CT arteriography or enhanced ultrasonography.

Key words: alpha-smooth muscle actin, hepatocellular carcinoma, neovascularity, non-triadal vessel, portal vein.

INTRODUCTION

Hepatocellular carcinoma (HCC) has been regarded as hypervascular on arterial angiography,[1–3] but early stage HCC often shows isovascular on angiography or dynamic computerized tomography (CT).[4–7] While both an increase in arterial blood flow and a decrease in portal blood flow are directly associated with the growth process of a small HCC,[8] little is known about the structures of tumor corresponding to arterial hypervascularity. Based on findings of imagings, a few authors[9–13] described that isovascular or even relatively

Correspondence: Kenji Ikeda, MD, Department of Gastroenterology, Toranomon Hospital, Toranomon 2-2-2, Minato-ku, Tokyo 105-8470, Japan. Email: ikedakenji@tora.email.ne.jp

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hypovascular tumors are hemodynamic characteristics of early stage HCC. Substantial and pathological evidence of isovascularity also remained unclear in a well-differentiated HCC.

Recently, we elucidated the relationship between arterial hypervascular characteristics and histological neovascular structure, shown by immunohistological staining using monoclonal alpha-smooth muscle actin (α-SMA) antibody.[14,15] Non-triadal vessels or unaccompanied vessels have proved to be characteristic and pathognomonic for HCC.[16] We described that there were two types of neovascular structure in the literature: type I vessel, principally found in well-differentiated HCC; and type II, exclusively found in moderate to poorly differentiated varieties.[14]

When type I vessels are detected in a nodular lesion, which is difficult to distinguish from regenerative nodules in ordinary histology, a small and well-differentiated HCC can be discriminated from a benign regenerative nodule. Although the appearance of a neovascular structure seems to be an essential event of early stage HCC, the origin of the vascular structure has been scarcely elucidated. The purpose of this study was to explain the nature and origin of a newly developed vascular structure found in well-differentiated HCC in relation to the implication from radiological images. To avoid sampling error in establishing the diagnosis of well-differentiated HCC in a fine-needle biopsy specimen, we only examined surgically resected specimens for ordinary histological characteristics and details of neovascular structure.

METHOD

Patients

Among 14 consecutive patients who underwent hepatic resection for well-differentiated HCC from 1991 to 1998, 11 patients were analyzed for hepatic imagings and immunohistochemical study of α-SMA. These patients had sufficient imaging diagnosis for hemodynamics of the tumor before surgical resection, including ultrasonography (US), dynamic CT, digital subtraction angiography (DSA), computerized tomographic arterial portography (CT-AP), computerized tomographic hepatic arteriography (CTHA), and intra-arterial carbon dioxide-enhanced ultrasonography (CO_2-US). The remaining three patients received local ablation therapy, chemotherapy or transcatheter arterial embolization prior to hepatic resection.

All the patients were males aged 53–70 years with a median age of 57 years. Two patients had hepatitis B surface antigen (HBsAg), six patients had second-generation antibody to hepatitis C virus (anti-HCV, enzyme-linked immunosorbent assay, Dainabot, Tokyo, Japan) and positive HCV-RNA. One patient had both HBsAg and anti-HCV, and the remaining two patients had neither. All the patients had liver cirrhosis, but no patient had decompensated cirrhosis with ascites or a history of encephalopathy. Median value for indocyanine green retention rate at 15 min (ICG R15) was 29%, and median platelet count was 109×10^3 counts/mm^3.

Imaging methods for hepatocellular carcinoma

Before angiographic study, conventional abdominal US, contrast enhanced-CT and DSA were performed in all patients. Two or more physicians carried out US studies using convex-array real-time scanners (LOGIQ 700, General Electric Yokogawa Medical Systems, Tokyo, Japan, and model SSA270A, Toshiba, Tokyo, Japan). Plain CT and contrast-enhanced CT were performed by a CT apparatus (HiSpeed Advantage SG, General Electric, Fairfield, CT, USA), with a scan time of 1 s and a thickness of 3–5 mm.

All 11 patients underwent CT-AP, CT-HA and CO_2-US at the time of DSA study. Selective hepatic intra-arterial DSA was performed by a DSA apparatus (Integris V3000, Philips, Netherlands) with a 0.7 mm focal spot, a 30 cm diameter image intensifier, and a 1024×1024 matrix. DSA with a selective catheterization distal to the common hepatic artery was performed using 5–15 mL of contrast medium (iohexol, Ominipaque, Daiichi Seiyaku, Tokyo, Japan) at an injection rate of 1.5–3.0 mL/s. When accessory or replaced hepatic arteries were found, we examined each hepatic artery through a super selective catheter at a rate of 0.7–1.5 mL/s. A sufficient amount of contrast medium and a carefully measured injection rate were used, avoiding laminar flow and uneven distribution of the contrast medium during each DSA study. Oxygen was administered to patients during the study to enable them to hold their breath for a period of 20 s. Tumor images obtained from DSA films were classified into two categories: a 'hypervascular' stain with sharply demarcated dense lesion adjacent to the site of a tumor, or an 'isovascular' stain with no apparent delineated lesion, which was considered to be of the same vascularity as the surrounding liver parenchyma.

To detect existence of the portal blood flow into the liver mass, CT-AP was carried out by injecting contrast medium through a catheter into the superior mesenteric artery. After 90 mL of diluted contrast medium was injected at a rate of 2.5–3 mL/s, CT were taken with a thickness of 3–5 mm throughout the liver. When a low-density area appeared at the location of the liver mass, suggesting paucity of portal blood flow, it was regarded as a characteristic finding for HCC rather than a regenerative nodule, and was judged to be a positive finding for HCC.

CT-HA was also performed using diluted contrast medium through a catheter placed in the common or proper hepatic artery. A total of 25–30 mL of diluted contrast medium was slowly infused at a rate of 0.8 mL/s. To detect a tiny difference in hepatic arterial blood flow to the tumor, the density of the site of the tumor nodule was judged compared with the surrounding liver tissue.

A dynamic US study was performed by injecting carbon dioxide gas through a catheter placed in the common hepatic or more distal arteries. Carbon dioxide was injected slowly, at a rate of approximately 0.5 mL/s, while monitoring US pictures. The total volume of injected gas varied from 2 mL to 5 mL. After an injection of carbon dioxide, if a tumor became hyperechoic

compared with the surrounding liver tissue, it was judged as a positive enhancement, indicating relative abundance of arterial blood flow. Conversely, if a tumor became hypoechoic compared with the surrounding liver, it was described as negative enhancement or a decreased arterial blood flow in the tumor nodule. As a rule, CT-AP study was performed prior to CO_2-US.

Characteristics of hepatocellular carcinoma

A solitary tumor nodule was found in each of the 11 patients on resected specimens and through ordinary diagnostic techniques, including US, CT, DSA, CT-AP and CT-HA. The median size of each primary tumor was 15 mm in diameter (range 10–22 mm). All the resected specimens were analyzed pathologically for the presence of satellite nodules and tumor extension to distal portal branches. Portal vein invasion was not found in all the resected specimens using macroscopic and histological examination. According to a published histological classification of HCC by Edmondson and Steiner,[17] all the tumors belonged to grade I HCC.

Histological diagnosis and immunohistochemical staining

At least two pathologists and one hepatologist made the diagnosis of HCC. Criteria and description by the International Working Party[16] for the features of well-differentiated HCC were taken into account in the establishment of diagnosis of liver cancer with an ordinary characterization of small HCC.

A part of each liver specimen was soaked in neutral formalin solution immediately after resection and fixed for 24–48 h. Paraffin-embedded fixed specimens were sliced into a thickness of 3 μm. A set of 400 serial thin sections of individual resected HCC specimen was made in each tumor nodule, which analyzed approximately 1.2 mm thickness of HCC tissue. After deparaffinization, immunohistological staining using α-SMA monoclonal antibody (N-terminal decapeptide of α-SMA, clone 1A4, Dako, Glostrup, Denmark) was performed with an indirect avidin-biotin complex method. Light counter staining for hepatocyte nuclei was made with hematoxylin.

When an α-SMA-positive tubular or ring-shaped structure was found unrelated to portal area on the immunohistological specimens, it was regarded as abnormal neovascularity, irrespective of the site in the liver. We therefore regarded such a ring-shaped structure shown by α-SMA staining as a non-triadal vessel and defined it as positive neovascularity. When this positive staining was found exclusively in portal areas, or an irregular, non ring-shaped sinusoidal lining was demonstrated in the liver specimens, they were regarded as non-specific findings for neovasculature.

The shapes of the non-triadal vessels were classified into two categories described previously.[14] Briefly, a type I vessel was characterized by a thin-walled, nuclei-poor and oval-shaped vessel, and type II as a thick-walled, nuclei-rich and slender-shaped vessel. The ratio of the major axis over the minor axis of an α-SMA positive vessel was usually two or less in type I, and three or more in type II.

RESULTS

Radiological characteristics of hepatocellular carcinoma (Table 1)

On intra-arterial DSA images, all patients showed an isovascular staining at the site of HCC detected by US and/or CT. Hypervascular staining was not found in the other parts of the liver. Although three patients showed a distinct low-density area, the other eight patients (72.7%) did not demonstrate any explicit low-density area on CT-AP study, suggesting no paucity of portal

Table 1 Radiological and immunohistochemical findings of 11 nodules of hepatocellular carcinoma

Case	Age/sex	Tumor size (mm)	Number of tumors	Digital subtraction angiography	Portal flow	Arterial flow	Thin-walled oval non-triadal vessel	Connection of neovascularity with pre-existing portal vein
1.	57M	10	solitary	isovascular	isovascular	decrease	positive	yes
2.	56M	12	solitary	isovascular	decrease	isovascular	positive	yes
3.	57M	14	solitary	isovascular	isovascular	decrease	positive	not detected
4.	61M	15	solitary	isovascular	isovascular	isovascular	positive	not detected
5.	53M	15	solitary	isovascular	isovascular	decrease	positive	yes
6.	63M	15	solitary	isovascular	decrease	isovascular	positive	yes
7.	62M	15	solitary	isovascular	isovascular	decrease	positive	yes
8.	70M	15	solitary	isovascular	decrease	isovascular	positive	yes
9.	59M	17	solitary	isovascular	isovascular	isovascular	positive	yes
10.	56M	20	solitary	isovascular	isovascular	isovascular	positive	yes
11.	54M	22	solitary	isovascular	isovascular	isovascular	positive	yes

blood flow in the tumor nodules. On CO_2-US study, isovascular enhancement was found in seven patients and negative enhancement (relative arterial paucity) was demonstrated in four patients. No patient showed positive enhancement on CO_2-US or CT-HA. These four tumors were 15 mm or less in size and showed a retained portal blood flow on CT-AP.

All 11 tumors showed isovascular staining on ordinary digital subtraction angiography, and four of the tumors showed negative enhancement on intra-arterial CO_2-US or CT-HA, suggesting arterial blood paucity.

Because entire tumor nodules did not show a feature of classical or advanced HCC with arterial hypervascularity, fine needle biopsy was required for establishment of diagnosis of HCC before surgical resection.

Histology of hepatocellular carcinoma and alpha-smooth muscle actin positive vessels

When the degrees of histological differentiation of HCC were classified according to Edmondson and Steiner,[17] all the resected HCC were classified as grade I. According to the criteria of histological classification by the International Working Party,[16] the findings of all tumor nodules coincided with the features of well-differentiated HCC. Each tumor in all 11 patients had increased cell density with a concentration more than twice as high as surrounding liver tissue. Fatty metamorphosis was found in three tumor nodules, pseudoglandular formation in four, interstitial tumor cell infiltration in six, enhancement of eosinophilic staining in cytoplasm in seven, and basophilic enhancement in nuclei in five. Mild to moderate degrees of nuclear atypism were also found in six tumors. Every tumor had a structure of portal triad within the nodule, consisting of portal vein, artery and bile duct. No tumors had fibrous capsular formation around them.

Alpha-SMA positive vascular structure was found in the cancerous parts of specimens in all 11 patients. All the positively stained vessels showed discrete, round or oval-shaped circular configuration. They also showed thin-walled vessels lined with scattered squamous nuclei, indicating type I vessels.[14] The number of the type I vessels found in a slice of the HCC specimens ranged from six to 83, with a median of 13. None of the HCC nodules had slender-shaped and thick-walled type II vessels with abundant round nuclei. Almost all of these immunohistochemically detected vessels were hardly identified as vascular or even ring-shaped structures on ordinary hematoxylin and eosin (HE) staining. The numbers of neovascular structure were few per high power field in eight patients and 5–10 in three patients.

Alpha-smooth muscle actin positive vessels in serial thin sections

In nine (81.8%) out of 11 HCC nodules, serial thin sections of the specimens showed that the thin-walled, oval non-triadal vessels were connected to portal veins in portal triads within well-differentiated cancer (Figs 1,2). This type of neovascular structure found in a well-differentiated HCC looked like a surviving portal vein among diminishing and disappearing arteries and bile ducts. We could not demonstrate the connection between unpaired vessels and portal triads within the tumor in the serial thin sections of the remaining two patients. Both of the latter two HCC nodules had a less number of α-SMA positive non-triadal vessels in the tumor tissues, and the origin of the existing non-triadal vessels remained unexplained from the limited number of tissues with a slice thickness of 1.2 mm.

In case 6 (Table 1), a small hepatic nodule 15 mm in diameter was detected in a routine check-up with US in a 63-year-old patient with HCV-related cirrhosis. Although DSA, CT-HA and CO_2-US could not demarcate arterial hypervascularity of the tumor, a relative decrease in portal blood flow was demonstrated by CT-AP. A histological finding from fine needle biopsy for the nodule showed a well-differentiated HCC. Surgically resected specimen also showed histology of a uniform well-differentiated variety of cancer with structures of portal triads within the HCC nodule. Alpha-SMA staining showed sparse unpaired vessels. Figure 1a–d illustrates microscopic findings of serial thin section of the resected HCC, showing that the unpaired vessels joined portal veins in original portal triads within the tumor nodule.

Case 7 (Table 1) was a 62-year-old man. HE staining for the resected specimen of the tumor showed a well-differentiated HCC with crowded nuclei, nuclear deviation to sinusoid, and interstitial invasion of the tumor cells. Immunohistochemistry with α-SMA disclosed the scattered existence of unaccompanied vessels in the tumor nodules. Figure 2a–d shows microscopic findings of serial thin sections of the α-SMA positive vessels, which were connected to normal portal veins in portal triad. Portal blood therefore fed the entire part of the tumor nodule, but arterial blood flow partly diminished with development of the non-triadal vessels.

Relationship between radiological findings and non-triadal vessels

Immunohistochemical study using α-SMA monoclonal antibody showed unaccompanied vessels in the all tumor tissues. Every tumor also had the structure of a portal triad within the tumor nodules. All but two HCC specimens showed a connection between the unaccompanied vessels and portal veins within portal triads. When the diagnosis of well-differentiated HCC was established, the joint between the unpaired vessels and original portal vein was found irrespective of the detailed findings of imagings.

Case 7 (Table 1), a 62-year-old man with HCV-positive cirrhosis, developed a solitary hepatic mass with a diameter of 15 mm. Routine check-up of his liver with US demonstrated a round hypoechoic mass in the left lobe of the liver (Fig. 3a). The nodule was isovascular on DSA, but slightly hypovascular on CO_2-US (Fig. 3b), which suggested relative paucity of arterial blood flow in the tumor nodule. CT-AP demonstrated

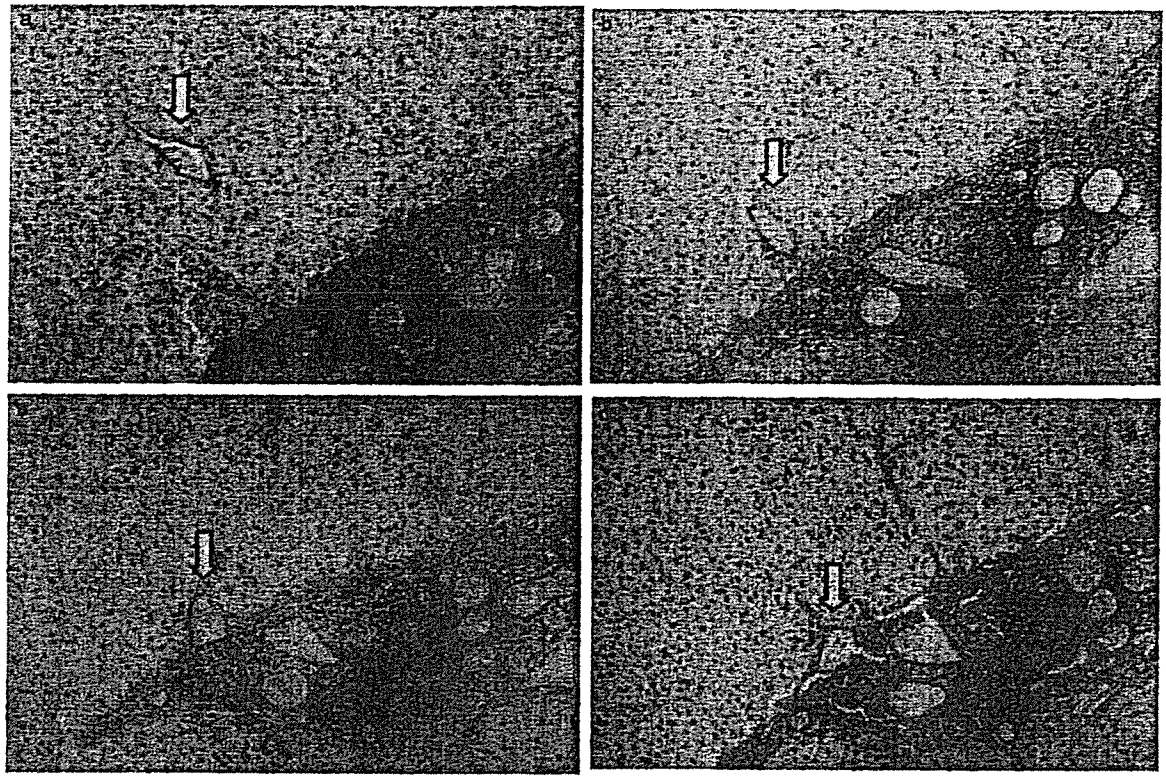

Figure 1 (a–d) Immunohistochemical staining of alpha-smooth muscle actin for serial thin sections of well-differentiated hepatocellular carcinoma in case 6. A non-triadal vessel joined a portal vein in an original portal tract. Arrows indicate a non-triadal vessel.

that there was a sufficient portal blood supply in the tumor nodule.

Alpha-smooth muscle actin in non-cancerous parts of liver

Unaccompanied vessel with positive α-SMA staining was not found in all the non-cancerous parts of the resected specimens. Alpha-SMA positive vascular structure was only found in the portal area as normal arterial and portal components of portal triad.

DISCUSSION

Murine monoclonal antibody α-SMA can recognize an actin isoform typical of smooth muscle cells and prevalent vascular smooth muscle cells.[18] Because α-SMA can delineate intrahepatic vessels including artery, portal vein, perisinusoidal cells and neovascular structure in HCC tissue,[15,19–24] we characterized specific features of neovascular structures in well-differentiated and moderately differentiated liver tumors, separately.[14] Briefly, non-triadal vessels found in HCC tissues proved to consist of two different varieties: type I vessels with round- or oval-shaped, thin-walled and sparse nuclear arrangement along the wall; and type II vessels with thick-walled, abundant nuclear arrangement and a much more slender and long-shaped structure (ratio of five times or more of the longest to the shortest diameter). Type I vessels were usually found in a well-differentiated HCC without hypervascularity, while type II non-triadal vessels were firmly associated with angiographic hypervascularity in moderately to poorly differentiated HCC.

In this study, we attempted to explore the origin of the non-triadal or unaccompanied vessels found in the early stage of HCC, through an immunohistochemical study of serial thin sections of HCC, using anti-α-SMA antibody. Anti-α-SMA antibody could successfully demonstrate the intrahepatic vascular structures containing smooth muscle: normal hepatic arteries, portal veins and intratumoral, non-triadal vessels. Although α-SMA staining was sometimes found lightly and irregularly in perisinusoidal parts of both cancerous and non-cancerous tissue, various forms of ring-shaped stains were only found in HCC. The non-triadal vessels found in a well-differentiated HCC were usually type I vessels with oval-shaped, thin-walled staining lined by scattered squamous nuclei. The current study, using serial thin sections of resected HCC specimens, disclosed that the oval-shaped vessels proved to be

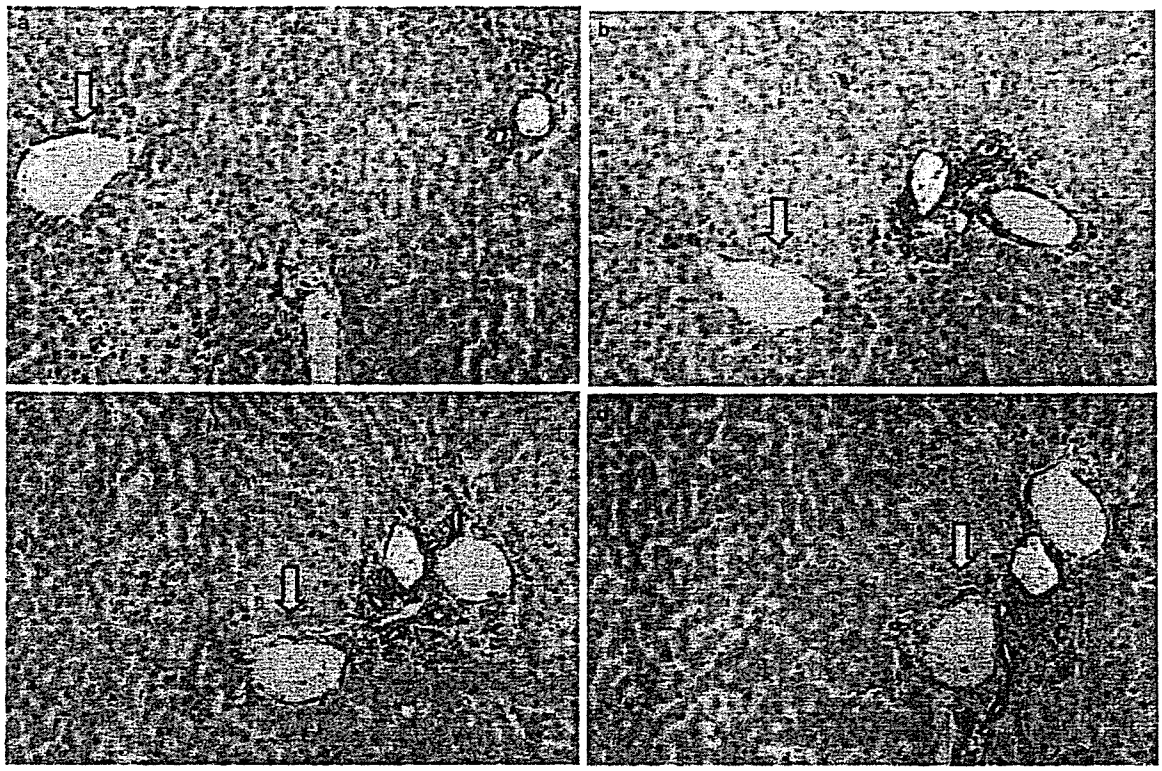

Figure 2 (a–d) Immunohistochemical staining of alpha-smooth muscle actin for serial thin sections of well-differentiated hepatocellular carcinoma in case 7. A non-triadal vessel is connected to a portal vein in a portal triad. Arrows indicate a non-triadal vessel.

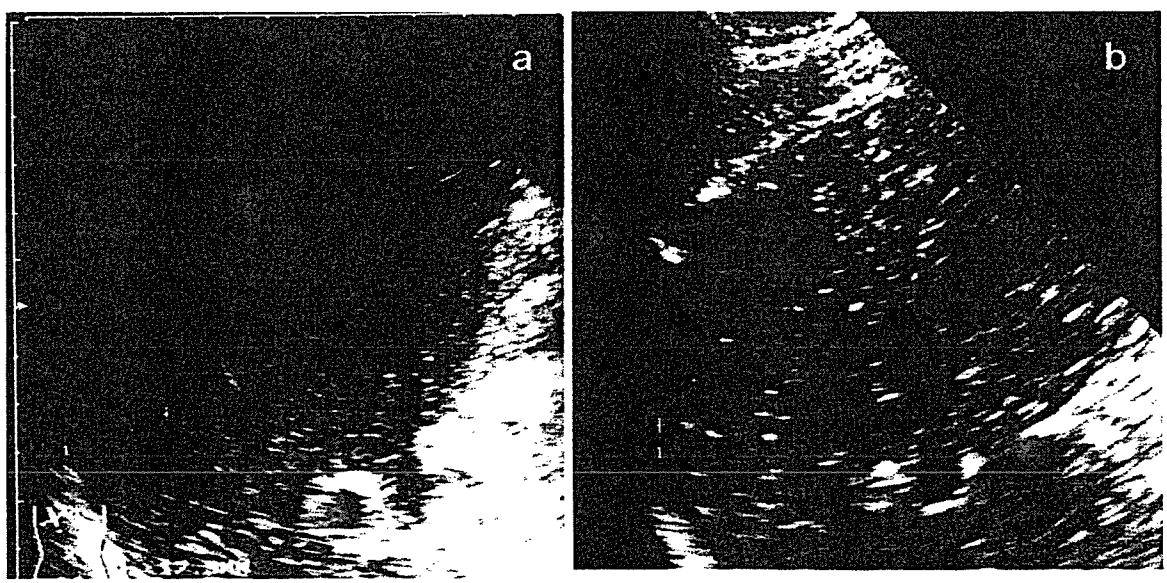

Figure 3 Ultrasonographic findings of a well-differentiated hepatocellular carcinoma (a) before and (b) after intra-arterial enhancement with carbon dioxide gas.

connected to portal veins in portal triad in HCC tissue. We could not demonstrate the origin of the unpaired vessels in well-differentiated HCC in only two of 11 specimens. One of the reasons for the failure of demonstration was scarcity of the number of unaccompanied vessels, and another reason was shortage of the slice thickness of total serial section. Unaccompanied vessels found in well-differentiated HCC were not connected with other vascular structures, including arteries in portal triads or hepatic veins. Although only the structure of portal veins definitely 'survived' from a portal triad, the reason why bile ducts and arteries 'perished' in a well-differentiated tumor was not clear.

Detection of non-triadal vessels using an immunohistological technique greatly helped in establishing diagnosis of well-differentiated HCC. Among various histological findings of well-differentiated HCC, demonstration of interstitial or stromal invasion[25] and detection of unpaired vessels were significantly valuable in the establishment of the diagnosis as HCC. The International Working Party[16] also supported the finding of non-triadal vessels in the diagnosis of HCC, and previous studies[14,23,24] described the value of the existence of specific vascular structure. Therefore, one of the important points of the current study was to explore the origin and pathogenesis of 'abnormal' non-triadal vessels found in a well-differentiated HCC. Type I vessels proved to originate from normal portal veins and this type of vessel did not meet the concept of the 'neovascular structure' of cancer. This type of neovascular structure looks like a surviving and remaining portal vein irrespective of surrounding tissue. Although this morphological study indicates that the 'abnormal' non-triadal vessel seems to be an abnormal portal vein only found in well-differentiated HCC, the possibility of the role of draining the vessel is not excluded completely.

The other significant implications shown in this study was an understanding of the imagings of blood flow at an early stage of HCC. A well-differentiated HCC sometimes shows shortage of hepatic arterial blood flow, which is demonstrated by CT-HA, CO_2-US, or both. During exploration of hepatic arterial dominance for small hepatic nodules in order to establish the diagnosis of HCC, varied modalities of imaging have been performed over the last decade.[8,26,27] Detailed analysis of imaging disclosed that some nodules of well-differentiated HCC proved to have hepatic arterial shortage instead of arterial hypervascularity.[9–11] The current study gave substantial explanation for the reason behind arterial blood paucity at an early stage of HCC. From the viewpoint of origin of non-triadal vessels, only portal veins survive and feed the tissue of well-differentiated HCC, while hepatic arteries and bile ducts diminish and disappear from the portal triads within HCC nodules. The ratio of hepatic arteries and portal veins in tumor nodules seems to result in findings of hepatic arterial paucity in sensitive tomographic imaging. The reason why each of the well-differentiated HCC nodules did not show arterial hypovascularity on imagings might be explained by sensitivity of each imaging modality, and by diversity and irregularity in the distribution of type I vessels. If the percentage of blood supply from pre-existing portal veins to an early stage of HCC was much more dominant than that from hepatic arteries, sensitive tomographic imagings would demonstrate arterial paucity in the tumor nodule. Moreover, actual blood flow per time and functional features of blood circulation in the vessels are still uncertain, and future studies should therefore be aimed at a pathophysiological aspect of hemodynamics of small and early HCC.

Non-triadal vessels should be evaluated correctly, not only in advanced HCC but also at an early stage of the tumor. The difference in angiographic vascularity might originate from an 'arterial character' in type II vessels and a 'portal character' in type I vessels. From the immunohistological results of vascular structure in HCC, only type II vessels seem to stand for 'neovascular' structure, which usually develops in any noticeable and hypervascular cancer of other organs. Type I vessels, which are almost exclusively found in a well-differentiated HCC, differed from the real definition of 'neovascular structure', because the non-triadal vessels proved to originate from pre-existing portal veins in this study. The realization of non-triadal vessel (unaccompanied or unpaired vessel) is still important in the establishment of diagnosis of early stage HCC, and in recognition of its hemodynamics. Two types of non-triadal vessels should be correctly identified not only for the study of radiological examination of liver cancer but also in the investigation of the angiogenetic process of HCC.

Although the exact reason for the solely surviving portal vein in a well-differentiated HCC tissue remains unknown, pathogenetic mechanisms are considered to differ significantly between type I and type II non-triadal vessels in HCC tissues. Type II angiogenesis is believed to depend on various kinds of angiogenetic factors including vascular endothelial growth factor and platelet derived growth factor. Our previous study[14] disclosed that type II vessels were firmly associated with angiographic hypervascularity, indicating substantial structure for arterial blood flow dominance in moderately to poorly differentiated HCC. Clinicopathological relationship and stimulating factors for angiogenesis have been studied recently,[28–31] and the pathogenesis and chorological process of characteristic vascular pattern should be investigated separately in well-differentiated HCC in the future.

In conclusion, non-triadal vessels found in early stage HCC originate from normal portal veins in portal triads, which constitute a 'pseudo-neovascular structure' in well-differentiated liver cancer. Importance of the unpaired vessels derived from portal veins include: (i) diagnostic help for histological exploration of well-differentiated variety of HCC; and (ii) substantial explanation of arterial blood paucity often found in detailed diagnostic imagings of early stage and small-sized HCC.

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p16 Promoter Hypermethylation in Human Hepatocellular Carcinoma with or without Hepatitis Virus Infection

Takashi Narimatsu[a] Akihiro Tamori[a] Noritoshi Koh[a] Shoji Kubo[b] Kazuhiro Hirohashi[b] Yoshihisa Yano[c] Tetsuo Arakawa[d] Shuzo Otani[c] Shuhei Nishiguchi[a]

Departments of [a]Hepatology, [b]Surgery, [c]Biochemistry and [d]Gastroenterology, Osaka City University Graduate School of Medicine, Osaka, Japan

Key Words
Hepatitis virus · Hepatocellular carcinoma · Methylation · *p16* · *p53*

Abstract
Background: Epigenetic alteration through methylation is one of the most important steps in carcinogenesis. However, the relation between hepatitis virus infection and epigenetic alterations is poorly understood. ***Methods:*** Sixteen patients without hepatitis B virus (HBV) and hepatitis C virus (HCV) and 35 patients with HBV or HCV who underwent liver resection for hepatocellular carcinoma (HCC) were studied. Mutation of *p53* was detected by direct sequencing. Methylation status of *p16* was evaluated in tumor and noncancerous liver tissues by methylation-specific polymerase chain reaction. ***Results:*** In HCC without HBV and HCV, *p53* mutations were detected in 5 (31%) of 16 HCCs. Methylation of *p16* promoter was detected in 2 (25%) of 8 moderately differentiated HCCs, 6 (75%) of 8 poorly differentiated HCCs, and none of 16 noncancerous tissue specimens. In HCC with HBV or HCV, *p53* mutations were detected in 8 (23%) of 35 HCCs. Methylation of *p16* promoter was detected in 2 (100%) of 2 well-differentiated HCCs, 13 (76%) of 17 moderately differentiated HCCs, 12 (75%) of 16 poorly differentiated HCCs, and 9 (26%) of 35 noncancerous liver tissue specimens. ***Conclusions:*** Our results suggest that hepatitis viruses might induce methylation of *p16* promoter in liver with chronic inflammation, before appearance of HCC.

Introduction

Hepatocellular carcinoma (HCC), one of the most prevalent malignancies in the world, is associated with chronic liver damage, such as infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). Basic research has suggested that hepatitis B X protein or hepatitis C core protein interacts with p53 or p21, both of which are cell-cycle modulators [1–3]. Overexpression of hepatitis B X or HCV core protein induces liver tumors in transgenic mice [4, 5]. In human liver, *p53* mutations are found in approximately 50% of advanced HCCs, irrespective of the presence of viral infection [6–8]. Cell-cycle disruption is one of the most important steps in human hepatocarcinogenesis. Recent studies show that epigenetic changes through methylation occur in the early stage of HCC [9,

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S. Nishiguchi
Department of Hepatology, Osaka City University Graduate School of Medicine
1-4-3 Asahimachi, Abeno-ku
Osaka 545-8585 (Japan)
Tel. +81 6 6645 3811, Fax +81 6 6645 3813, E-Mail snishiguch@med.osaka-cu.ac.jp

10]. Aberrant hypermethylation of CpG islands, CpG-dinucleotide-rich areas located mainly in the promoter regions of many genes, has been implicated in the transcriptional silencing of tumor suppressor genes in cancer [11–13]. In particular, *p16*, a cyclin-dependent kinase inhibitor, has been associated with promoter hypermethylation and decreased protein expression in cancers, including HCC [14].

In Japan, nearly all patients with HCC have been infected with hepatitis virus. Molecular changes in HCC have not been reported to differ between the presence and absence of hepatitis virus. However, delineation of differences between HCC with viral infection and HCC without viral infection may provide new insights into the mechanism of hepatocarcinogenesis. In this study, we investigated methylation status of the *p16* promoter gene and *p53* mutations in HCCs with and those without hepatitis virus infection. Also, we examined *p16* promoter methylation status in noncancerous liver.

Subjects and Methods

Samples

Fifty-one HCC samples were obtained during surgery at Osaka City University School of Medicine. One portion of each specimen was frozen in liquid nitrogen immediately after resection and stored at −80° until analysis (table 1).

Total RNA and DNA were extracted from this portion by conventional methods. Sixteen samples were not infected with HBV or HCV (mean age 61.9 years; 9 men and 7 women; 2 autoimmune hepatitis, 1 primary biliary cirrhosis, 3 excessive alcohol drinkers, 7 habitual alcohol drinkers, and 3 unknown etiology for liver disease).

Thirty-five HCCs were infected with HBV or HCV (mean age 60.0 years; 33 men and 2 women; 9 HBV-positive, 25 HCV-positive, and 1 positive for both viruses).

The samples were histopathologically examined and classified as well-differentiated HCC (n = 2), moderately differentiated HCC (n = 25), and poorly differentiated HCC (n = 24). The noncancerous tissues were classified as cirrhosis (n = 19) and noncirrhosis (n = 16). This study was performed in accordance with the Helsinki Declaration of 1975 (1983 revision) and was approved by the ethics committee of Osaka City University Medical School.

Methylation-Specific Polymerase Chain Reaction

Bisulfite modification of genomic DNA was performed as described by Herman et al. [15]. Briefly, 1 μg of DNA was denatured with NaOH, and 10 m*M* hydroquinone and 3 *M* sodium-bisulfite were added. The sample was incubated at 50° for 16 h. Modified DNA was purified with the use of Wizard DNA purification resin, followed by ethanol precipitation. DNA methylation patterns were determined by chemical modification of the unmethylated cytosines to uracil and subsequent polymerase chain reaction (PCR) using primers specific for either methylated or modified unmethylated DNA. Primer sequences were 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (upper primer) and 5′-CAACCCCAAACCACAACCATAA-3′ (lower primer) for unmethylated *p16* and 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (upper primer) and 5′-GACCCCGAACCGCGACCGTAA-3′ (lower primer) for methylated *p16* [16]. The PCR amplification of modified DNA samples consisted of 1 cycle of 95° for 10 min; 35 cycles of 95° for 45 s, 53° for 45 s, and 72° 45 s; and 1 cycle of 72° for 2 min. Ten microliters of each PCR product was loaded directly onto nondenaturing 5% polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet illumination.

Table 1. Main clinicopathologic features

Male/female	42/9
Mean age, years	60.6
HBsAg (+)	9
Anti-HCV (+)	25
HBsAg (+) and anti-HCV (+)	1
Anti-HCV (−) and HBsAg (−)	16
Tumor differentiation	
Well/moderately/poorly	2/25/24
Tumor diameter (<3 cm/>3 cm)	31/20
Vascular invasion	17 (33%)
Intrahepatic metastasis	15 (29%)

Semiquantitative Reverse-Transcription PCR Analysis

To investigate *p16* mRNA expression, we performed reverse-transcription PCR (RT-PCR) with the RNA of tumors and noncancerous lesion. Briefly, 1 μg of RNA was used as a template to generate complementary DNA by random hexamers and reverse transcriptase. The complementary DNA was used for PCR amplification. Primer sequences were 5′-CCACCCCGCTTTCGTAGTTTT-3′ (upper primer) and 5′-TGCGAGGCTCGCAAGAAAT-3′ (lower primer) for *p16* and 5′-CCTCGCCTTTGCCGATCC-3′ (upper primer) and 5′-GGATCTTCATGAGGTAGTCAGTC-3′ (lower primer) for β-actin. The PCR amplification consisted of 1 cycle of 95° for 12 min; 35 cycles of 95° for 30 s, 51° for 1 min, and 72° for 30 s; and 1 cycle of 72° for 3 min *(p16)*; 1 cycle of 94° for 3 min; 33 cycles of 95° for 30 s, 60° for 1 min, and 72° for 30 s, and 1 cycle of 72° for 3 min (β-actin). Ten microliters of each PCR product was loaded directly onto nondenaturing 5% polyacrylamide gels and the gels were stained with SYBR Greene (BioWhittaker Molecular Applications, Rockland, USA) according to the manufacturer's protocol. The intensity of the bands was quantified by densitometry.

Detection of p53 *Mutations*

We directly sequenced exon 5–8 of the *p53* genes, in which 98% of *p53* mutations had occurred, in the 51 tumors. One hundred nanograms of genomic DNA was subjected to 35 PCR cycles (94, 55, and 72° for 0.5, 0.5, and 1 min, respectively) with rTaq DNA polymerase. Double-stranded DNA was sequenced using dideoxy chain termination technique. Gel electrophoresis and DNA sequencing were performed with a DNA sequencing system.

Statistical Analysis

The factors in the two groups were examined by χ^2 test.

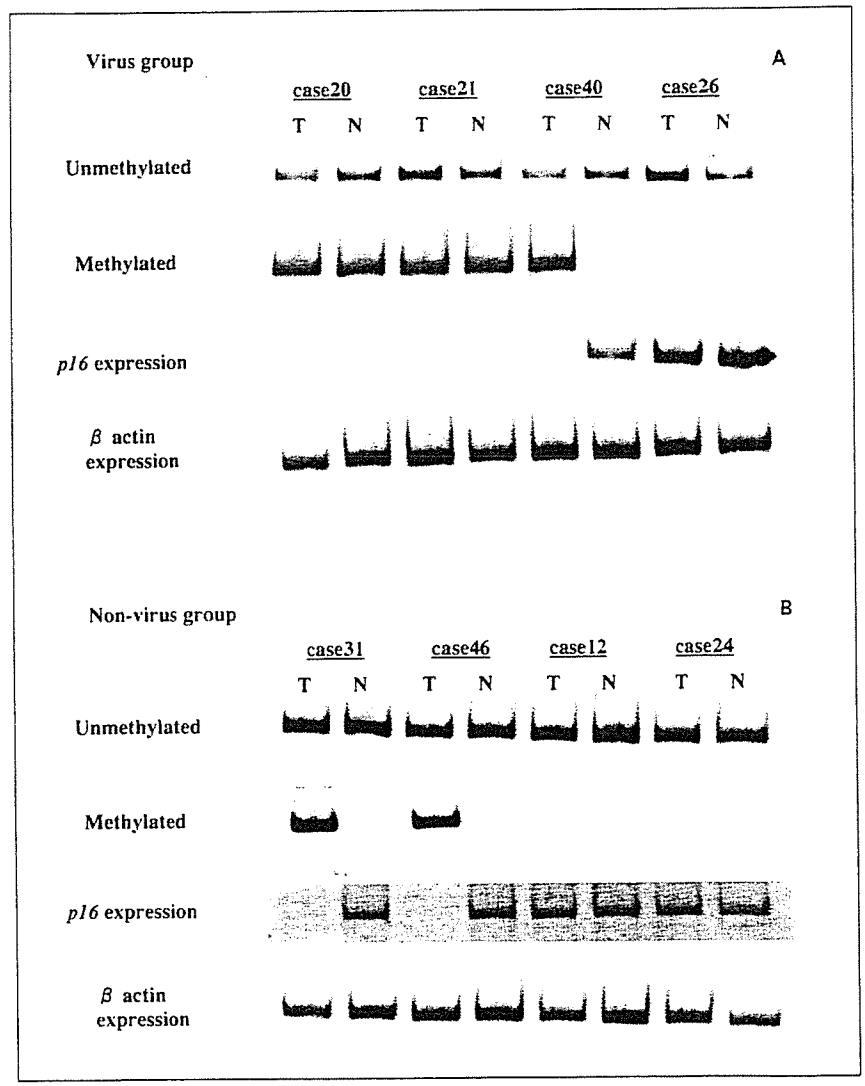

Fig. 1. Correlation between *p16* methylation and gene expression. Representative methylation-specific polymerase chain reaction results are expressed as unmethylated *p16*-specific bands or methylated *p16*-specific bands. Shown are the results of RT-PCR studies for *p16* and β-actin (used as an integrity control). Each results of HCC and noncancerous liver tissue in a case with virus infection (**A**) and without virus infection (**B**). T = HCC tumor tissue; N = noncancerous liver tissue.

Results

Methylation of p16 *Promoter and Expression of* p16 *Gene* (fig. 1, table 2)

Methylation of *p16* promoter was detected in 35 (69%) of 51 HCCs and 9 (18%) of 51 noncancerous tissue samples. *p16* expression was not detected in 31 (61%) of 51 HCCs and 9 (18%) of 51 noncancerous tissue samples. *p16* was not expressed in 29(83%) of 35 HCCs and 7 (78%) of 9 noncancerous tissue samples with methylation of *p16* promoter. In the other samples with *p16* methylation, expression of *p16* was low, compared to that of samples without methylation.

Methylation of *p16* promoter was detected in 8 (50%) of 16 HCCs without hepatitis virus infection. In brief, *p16* methylation was detected in 2 (25%) of 8 moderately differentiated HCCs and 6 (75%) of 8 poorly differentiated HCCs in this group (fig. 2). Methylation of *p16* promoter was not detected in any of 16 noncancerous tissue samples without hepatitis virus infection. Methylation of *p16* promoter was detected in 27 (77%) of 35 HCCs with HBV or HCV. In brief, *p16* methylation was detected in 5 (56%) of 9 HCCs with HBV, 21 (84%) of 25 HCCs with HCV, and 1 HCC with both viruses. According to histopathological classification, methylation of *p16* promoter was detected in 2 (100%) of 2 well-differentiated HCCs, 13

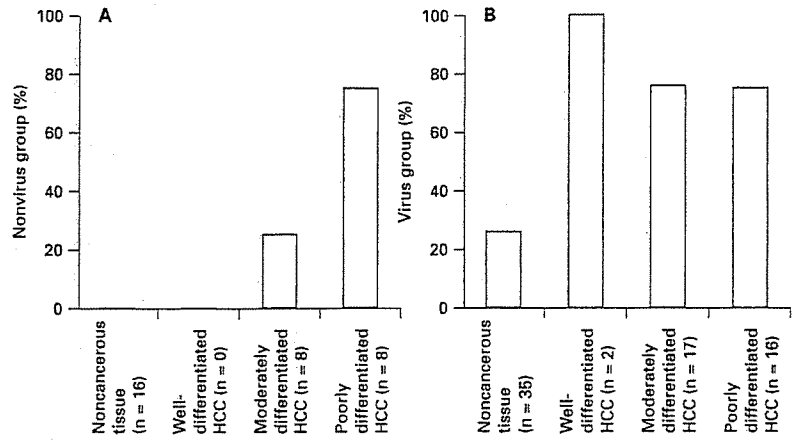

Fig. 2. Frequency of methylation studies in cases with (**A**) and without (**B**) virus infections.

Table 2. Methylation of *p16* promoter

	Nonvirus group (n = 16)	Virus group (n = 35)
Noncancerous tissue		
Noncirrhosis	0/11 (0%)	3/21 (14%)
Cirrhosis	0/5 (0%)	6/14 (43%)
HCC	8/16 (50%)	27/35 (77%)
Vascular invasion		
Positive	3/4 (75%)	8/13 (62%)
Negative	5/12 (42%)	19/22 (86%)
Intrahepatic metastasis		
Positive	1/4 (25%)	6/11 (55%)
Negative	7/12 (58%)	21/24 (88%)
p53 mutation		
Positive	1/5 (20%)	5/8 (63%)
Negative	7/11 (64%)	22/27 (81%)

Table 3. *p53* mutations in HCC

	p53 mutation	
	codon	amino acid change
Nonvirus group (n = 16)	155 ACC to AAC	Thr to Asn
HBsAg (–) and anti-HCV (–)	221 GAG to GAA	Glu to Gul
	184 GAT to TAT	Asp to Thr
	159 GCC to AGC	Ala to Ser
	225 TCT to TTT	Ser to Phe
Virus group (n = 35)	132 AAG to TTG	Lys to Leu
HBsAg (+) or anti-HCV (+)	133 ATG to TTG	Met to Leu
	272 GAG to GTG	Glu to Val
	189 GCC to GTC	Ala to Val
	220 TAT to TGT	Tyr to Cys
	123 TAT to TTC	Tyr to Phe
	275 TGT to TAT	Cys to Tyr
	292 insertion	

(76%) of 17 moderately differentiated HCCs, and 12 (75%) of 16 poorly differentiated HCCs (fig. 2). Methylation of *p16* promoter was detected in 9 (26%) of 35 noncancerous tissue samples with viral infection. In brief, *p16* methylation was detected in 3 (33%) of 9 HCCs with HBV and 6 (24%) of 25 HCCs with HCV. According to histopathological classification, methylation of *p16* promoter was detected in 6 (43%) of 14 regions of hepatic cirrhosis and 3 (14%) of 21 regions of chronic hepatitis ($p = 0.581$).

There was no significant difference in methylation of *p16* promoter between HCC without hepatitis virus infection and HCC with it ($p = 0.525$). On the other hand, the frequency of methylation of *p16* promoter was significantly higher in noncancerous tissue with hepatitis virus infection than in tissue without such infection ($p = 0.025$).

Mutation of p53

p53 mutations were detected in 5 (31%) of 16 HCCs without HBV and HCV (table 3). Among HCCs with HBV or HCV, *p53* mutation was detected in 8 (23%) of 35

HCCs (table 2). There is no significant relation between *p53* mutation and hepatitis virus infection. We contrasted *p53* mutation with methylation of *p16* promoter. Methylation of *p16* promoter was detected in 6 (46%) of 13 HCCs with *p53* mutation and 29 (76%) of 38 HCCs without *p53* mutation ($p = 0.043$).

Discussion

In this study, we showed that *p16* promoter was methylated in HCC, irrespective of the presence or absence of viral infection. Previous studies have documented methylation of *p16* promoter in many kinds of cancers, including lung cancer, prostate cancer, osteosarcoma, and HCC. Recently, Roncalli et al. [16] reported that cell-cycle regulator genes are frequently inactivated by methylation in HCC. Methylation of *p16* has been detected by Matsuda et al. [14] (82.7%), Liew et al. [17] (62.5%), Kaneto et al. [18] (72.7%), and Roncalli et al. [16] (82%). None of these reports described the relation between hepatitis virus infection and methylation. We found that *p16* gene promoter was methylated in 77% of HCCs with hepatitis virus infection and 50% of HCCs without such infection. In this study, no significant difference in *p16* methylation was found between the HBV-positive group and HCV-positive group. Zhong et al. [19] suggested that GSTP1 inactivation by hypermethylation is associated with HBV-related HCC. Shen et al. [20] reported that environmental exposures might induce gene methylation in HCC. There were some reports that viral infection might induce DNA methylation. For example, in Epstein-Barr virus-infected cells, methylation of DNA was demonstrated and in adenovirus-infected tumor, host DNA was frequently methylated [21, 22]. We believe that methylation status of gene promoter may be influenced by etiology, including hepatitis viral infection. The relation between hepatitis virus infection and gene methylation thus requires further study.

Recent reports suggest that gene methylation has already occurred in precancerous lesions in many types of organs [23]. Roncalli et al. [16] reported that one of the cell-cycle-related genes was frequently inactivated by methylation in hepatic cirrhosis. Kaneto et al. [18] detected methylation of *p16* in liver cirrhosis and chronic hepatitis associated with HBV or HCV infection. This group of investigators found no gene methylation in primary biliary cirrhosis, autoimmune hepatitis, drug-induced liver disease, fatty liver, or normal liver tissue in patients whose livers were negative for hepatitis virus infection. They suspected that epigenetic alterations in liver might be related to hepatitis virus infections. In our study, *p16* methylation was also detected in noncancerous liver tissue with hepatitis virus infection but not in liver tissue without hepatitis virus infection. However, in the patients without viral infection, *p16* methylation was detected in 25% of moderately differentiated HCCs and 75% of poorly differentiated HCCs. These results suggest that methylation is an early event in hepatocarcinogenesis in patients with hepatitis virus infection and is related to less differentiation of HCC in patients without virus infection.

Mutation of *p53* is one of the most common molecular alterations in human cancers. It is well known that *p53* mutation is not an early event in hepatocarcinogenesis. In this study, the detection rate of *p53* mutations in HCC without hepatitis virus was similar to that in HCC with hepatitis virus. These results suggest that *p53* mutation may be a common pathway in hepatocarcinogenesis, both in hepatitis-virus-positive and hepatitis-virus-negative patients. In this study, the frequency of methylation of *p16* promoter was significantly higher in HCC without *p53* mutation than in HCC with such mutation. This result suggests that intragenic and epigenetic alterations occur independently in HCC and support the notion that different mechanisms can lead to hepatocarcinogenesis.

In conclusion, our results suggest that *p53* mutations occur in both HCC without hepatitis virus and HCC with hepatitis virus. Methylation of *p16* promoter may be related to less differentiation of HCC without hepatitis virus. Hepatocarcinogenesis may progress through different pathways, depending on the presence or absence of viral infection.

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APPLIED NUTRITIONAL INVESTIGATION

Problems in Serum Albumin Measurement and Clinical Significance of Albumin Microheterogeneity in Cirrhotics

Akiharu Watanabe, MD, Shohei Matsuzaki, MD, Hisataka Moriwaki, MD, Kazuyuki Suzuki, MD, and Shuhei Nishiguchi, MD

From the Third Department of Internal Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan; the First Department of Gastroenterology, Tokai University, Isezaki, Japan; the First Department of Internal Medicine, Gifu University, Gifu, Japan; the First Department of Internal Medicine, Iwate Medical University, Morioka, Japan; and the Third Department of Internal Medicine, Osaka City University, Osaka, Japan

OBJECTIVES: To clarify problems with the determination of serum albumin levels, the definition of hypoalbuminemia, and the implications of microheterogeneity of albumin, serum albumin was measured by using dye-binding methods and the authentic method (immunoassay) in patients with liver cirrhosis and healthy subjects.

METHODS: We enrolled 103 patients with liver cirrhosis and 36 healthy subjects. Serum albumin levels were analyzed by immunoassay and the bromcresol green and bromcresol purple methods. Oxidized albumin and glycoalbumin were determined by high-performance liquid chromatography.

RESULTS: In cirrhotic patients, serum albumin levels measured by the bromcresol green method was about 0.2 g/dL higher than that by immunoassay. Serum albumin levels measured by the bromcresol purple method also was higher in cirrhotic patients than those measured by immunoassay and varied widely. In addition, extensive variation was found across serum albumin levels determined by the bromcresol green method at individual institutions (five university hospitals) and those determined by immunoassay at a contract laboratory. The percentages of oxidized albumin and glycoalbumin within total serum albumin increased with progression of liver disease. Further, an increase in oxidized albumin led to an increase in the albumin level as measured by the bromcresol purple method.

CONCLUSION: These results show that adequate assessment of the pathophysiology and prognosis of patients with liver cirrhosis and the efficacy of treatment is not possible with dye-binding methods for determination of serum albumin. Further, the conventional definition of hypoalbuminemia as a serum albumin level of 3.5 g/dL or lower should be reconsidered, and the clinical implications of qualitative changes in albumin should be investigated in consideration of the microheterogeneity of albumin, such as oxidized albumin and glycoalbumin. *Nutrition* 2004;20:351–357. ©Elsevier Inc. 2004

KEY WORDS: serum protein, liver disease, oxidized albumin, glycoalbumin, advanced glycation end products

INTRODUCTION

Over the years, serum albumin has been commonly measured as a laboratory test to identify malnutrition[1–3] and hepatic function disorder.[4] When branched-chain amino acid (BCAA) preparations are used to improve hypoalbuminemia in cirrhotic patients,[5–8] it is important to repeatedly measure the albumin level to detect slight changes. Thus, it is necessary to measure serum albumin levels more accurately and to establish a global standard for the determination of serum albumin with minimal differences between institutions.

Currently, serum albumin is determined in Japan by using bromcresol green (BCG), which is used at 91% of levels used at medical institutions (reference value, 3.8–5.3 g/dL), and bromcresol purple (BCP), which is used at 8% of levels used at medical institutions (reference value, 3.6–5.1 g/dL). However, a problem with these two methods is the large coefficient of variation across institutions (4.5–17.6%).[9]

Therefore, a serum albumin level in a patient diagnosed with hypoalbuminemia at one institution may be considered to be normal at another institution, resulting in delay of treatment to improve the malnutrition status. The classification system developed by Child and Trucotte[10] often is used to assess the severity of cirrhosis, in which serum albumin is included as one of five parameters (3.5 g/dL is used as a reference value for classification). In cirrhotic patients, a serum albumin level of 3.5 g/dL has been used for diagnosis of hypoalbuminemia. In addition, this albumin level often has been used for pathophysiologic evaluation and to determine when to start treatment. It has been reported that improvement of hypoalbuminemia by treatment with BCAA supplementation and the effect on the prognosis depend on when treatment is started and that the efficacy of BCAA decreases as the patient's albumin level decreases.[11]

In Child's classification, serum albumin is measured by a color reaction that is performed with 2-(4-hydroxyazobenzene)benzoic acid.[11] Because this method shows poor specificity for albumin,

Correspondence to: Akiharu Watanabe, MD, Third Department of Internal Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan. E-mail: awat@toyama-mpu.ac.jp

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38 y ago the reference value of serum albumin was 4.6 to 6.7 g/dL, which is considerably higher than that used at present. Therefore, the reference value of 3.5 g/dL used 38 y ago is considered to have very different clinical implications from the serum albumin level of 3.5 g/dL obtained by using current, more specific methods. However, even today, the serum albumin level of 3.5 g/dL in Child's classification is being used worldwide regardless of the analytical method.

Albumin used to be considered a homogeneous component as compared with other serum proteins. However, recent advances in protein chemistry and molecular biology have shown that albumin has microheterogeneity because oxidized and reduced forms of albumin[12,13] and glycoalbumin[14] have been identified. The biological and clinical implications of these molecular species remain unclear. In the current clinical scene, physicians are interested only in whether the serum albumin level is high or low. In the future, in patients with various diseases showing quantitative changes in albumin level, qualitative analysis also should be performed to clarify the kinetics of molecular species and various functions of albumin and for diagnosis, pathophysiologic analysis, and disease management.

We performed a multicenter study in patients with liver cirrhosis to investigate differences in serum albumin levels as a function of the analytical method and the institution. We also investigated changes in the levels of oxidized albumin (non-mercaptalbumin), reduced albumin (mercaptalbumin), and glycoalbumin to clarify problems with the analytical methods of serum albumin and the implications of albumin microheterogeneity.

MATERIALS AND METHODS

Subjects

Subjects were selected from among patients who visited five institutions specializing in hepatic disease during the 3 mo from January 15 to March 31, 2000. Subjects included 103 patients with cirrhosis due to any cause (inpatients and outpatients), 75 y or younger, in whom the serum albumin level was 2.2 to 4.9 g/dL by the BCG method; control subjects were 36 healthy subjects in whom the serum albumin level was considered to be within the normal range (3.6–4.9 g/dL by immunoassay; Table I). Diagnosis of cirrhosis was based on the histologic findings of liver biopsy, except for patients who were diagnosed based on blood biochemistry or imaging of the liver. Cirrhosis attributable to hepatitis C was the most frequent (68.9%), followed by hepatitis B (9.7%). Patients with alcoholic cirrhosis comprised 11.6%. Child's classification showed 49.5% for Child A, 27.2% for Child B, and 14.6% for Child C. Hepatocellular carcinoma that was not considered to affect the pathophysiology of cirrhosis was observed in 43 patients (41.7%). Complications were defined as esophageal or gastric varices, ascites, jaundice, and hepatic encephalopathy. When at least two of these four conditions were present concurrently, the patient was documented as "with complications." Patients with diabetes mellitus were identified at the time of blood sampling by asking whether oral hypoglycemic agents or insulin were used. BCAA preparations (enteral nutrition or amino acid preparations) were being administered to 48 patients (46.6%), but only 12 patients (11.7%) were receiving human albumin fluids. Informed consent was obtained in writing or orally from all patients enrolled in this study.

Blood samples were collected from the cubital vein under fasting conditions early in the morning. Immediately after blood collection, sera were separated and divided into samples for in-house testing and for testing at SRL Medisearch Inc. (SRL; Tokyo, Japan; two 0.5-mL tubes and one 2.8-mL tube). Samples for in-house testing were tested immediately by the BCG method at each institution. The following BCG kits and reference standards were used: institution A—Cica Auto ALB (Kanto Kagaku Company, Tokyo, Japan), ANA Serum ALB-TP Standard Solution (bovine; Daiichi Pure Chemical Company, Ltd., Tokyo, Japan); institution B: ALB Reagent A, ALB Standard Solution (human; International Reagent Corp., Kobe, Japan); institution C: Clinimate, ANA Serum ALB-TP Standard Solution (bovine; Daiichi Pure Chemical Company); institution D: Wako Alb Kit, Protein Standard Serum (human; Wako Pure Chemical Industries, Ltd., Tokyo, Japan); and institution E: Clinimate, ANA Serum ALB-TP Standard Solution (bovine; Daiichi Pure Chemical Company). The reference (normal) ranges for serum albumin were 4.3 to 5.4 g/dL at institution A, 3.9 to 4.9 g/dL at institution B, 3.5 to 5.0 g/dL at institution C, 4.1 to 5.0 g/dL at institution D, and 4.2 to 5.3 g/dL at institution E (thus, the lower limit of the reference range measured by the BCG method varied widely from 3.5 to 4.3 g/dL). All serum samples for testing by SRL were transferred from the individual institutions to a laboratory in Tokyo and stored in a freezer at −80°C. After all serum samples were received (samples were stored in the freezer for 3 mo at the longest), SRL thawed the frozen samples and measured albumin levels as described below.

TABLE I.

BASELINE CHARACTERISTICS OF PATIENTS

	Healthy Controls	Patients with liver cirrhosis
Patients, *n*	36	103
Male/female	22/14	60/43
Age (y)*	55.4 ± 10.1	63.7 ± 8.8
Etiology		
HCV related		71
HBV related		10
Alcohol		12
Other/unknown		6/4
Child class		
A		51
B		28
C		15
Unknown		9
Hepatocellular carcinoma		43
None		60
Complications†		31
None		72
Diabetes		18
None		85
n Patients with serum creatinine levels >1.0 mg/dL/*n* patients determined‡		1/52

* Mean ± standard error.
† Patients with at least two of the following complications: esophageal or gastric varices, ascites, jaundice, and hepatic encephalopathy.
‡ Levels were measured 2 wk before and after albumin determination.
HBV, hepatitis B virus; HCV, hepatitis C virus

Methods

The methods for albumin determination used at SRL were immunoassay (nephelometric immunoassay: N Antiserum to Human Albumin and N Protein Standard SL, Dade Behring Marburg GmbH, Marburg, Germany; reference concentration, 3.9–4.9 g/dL),[15] which was the reference method, the BCG method (Wako Alb Kit and Protein Standard Serum, human; Wako Pure Chemical Industries, Ltd.; reference concentration, 4.0–5.0 g/dL),[16] and the

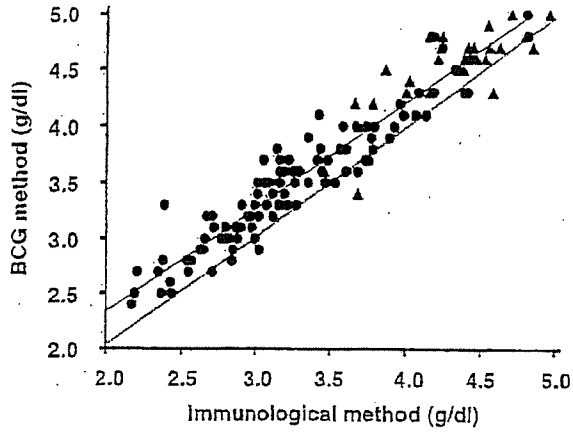

Fig. 1. Correlation of serum albumin levels measured by the BCG method at individual institutions and by immunoassay at the contract laboratory. Serum albumin levels as measured by the BCG method were higher in the measurement range than were those measured by immunoassay. A serum albumin level of 3.5 g/dL when measured by immunoassay corresponded to approximately 3.7 g/dL when measured by the BCG method. BCG, bromcresol green (3,3′,5,5′-tetra-bromo-*m*-cresolsulfonphthalein); circles, patients with liver cirrhosis (n = 103); triangles, healthy controls (n = 36); solid line, regression equation: $y = 0.93x + 0.47$ ($R^2 = 0.925$); dotted line, assuming that the same measured value is obtained with both methods at a correlation coefficient of 1.0 (regression equation: $y = x$).

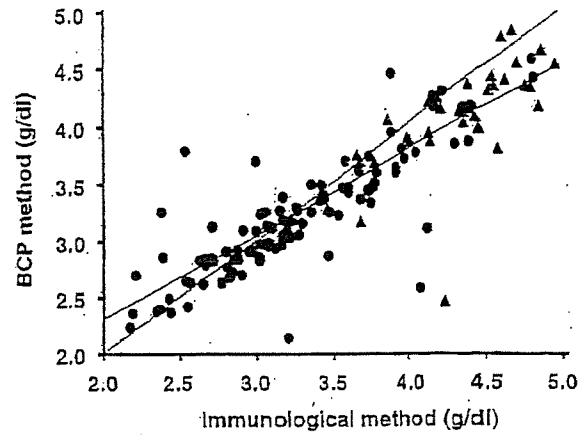

Fig. 2. Correlation of serum albumin levels measured by the BCP method at individual institutions and by immunoassay at the contract laboratory. In most patients with hypoalbuminemia, serum albumin levels as measured by the BCP method were higher than those measured by immunoassay. In normal control subjects in whom the serum albumin levels were within the normal range, serum albumin levels measured by the BCP method were lower than those measured by immunoassay. BCP, bromcresol purple (5,5′-dibromo-*o*-cresolsulfonphthalein); circles, patients with liver cirrhosis (n = 103); triangles, healthy controls (n = 36); solid line, regression equation: $y = 0.74x + 0.83$ ($R^2 = 0.667$); dotted line, assuming that the regression equation is $y = x$ at a correlation coefficient of 1.0. The regression lines with confidence intervals for healthy controls and cirrhotic patients are $y = 0.77x + 0.71$ ($R^2 = 0.46$) and $y = 0.71x + 0.94$ ($R^2 = 0.52$), respectively.

BCP method, which is highly specific for albumin (Eiken EA Test ALB, TP-ALB Reference Standard for Autoanalyzer, and Eiken Standard Solution, human; Eiken Chemical Company, Ltd., Tokyo, Japan; reference concentration not specified).[17] Oxidized albumin and glycoalbumin were determined by high-performance liquid chromatography.[18,19] The percentage of each molecular species in the total serum albumin level measured by immunoassay was calculated as follows:

$$\text{oxidized albumin percentage (\%)} = \text{oxidized albumin}/(\text{oxidized albumin} + \text{reduced albumin}) \times 100$$

$$\text{glycoalbumin percentage (\%)} = \text{glycoalbumin}/(\text{glycoalbumin} + \text{non-glycoalbumin}) \times 100$$

The correlation of albumin levels between analytical methods and the correlation between the oxidized albumin percentage and the glycoalbumin percentage were tested by simple regression analysis by calculating a linear regression curve. Comparison of the mean values between two groups was performed with Student's t test, with $P = 0.05$ indicating statistical significance. Comparison of the mean values across three groups was performed by analysis of variance followed by Bonferroni's multiple test (Statview 4.54.0.0, 1996, Abacus Concepts, Inc., Berkeley, CA, USA), with $P = 0.05$ considered statistically significant.

RESULTS

Figure 1 shows the correlation between the serum albumin levels measured at the contract laboratory by immunoassay (the reference method for determination of serum albumin) and the BCG method (the most commonly used method in Japan). The serum albumin level measured by BCG ranged from 2.2 to 4.9 g/dL and tended to show higher values than the level measured by immunoassay. Figure 2 shows the correlation between serum albumin levels measured by immunoassay and the BCP method. In cirrhotic patients, the values yielded by the BCP method also were generally higher than the immunoassay values. In the control subjects, the BCP values were lower than the immunoassay values. The differences between the two methods were particularly larger in the cirrhotic patients. In general, values measured by the BCP method showed greater variation than those by the BCG method.

Using 48 serum samples (3 to 13 samples per institution) whose albumin levels measured by immunoassay ranged from 3.0 to 3.5 g/dL without significant differences in the mean values at the individual institutions, the difference between the BCG data at the individual institutions and the immunoassay data at the contract laboratory was calculated (Figure 3). At some institutions the mean value by the BCG method was higher (sites B, D, and E) or lower (sites A and C) than that by immunoassay. The coefficient of variation among institutions was 15.7%. This result suggests that a serum sample containing albumin at a level of 3.5 g/dL when measured by immunoassay may show a range of 3.3 to 3.9 g/dL when measured by the BCG method.

The possibility of a relation between serum albumin level and development of complications was investigated. Of 17 patients in whom the serum albumin level was higher than 3.8 g/dL when measured by immunoassay at the contract laboratory and by the BCG method at the individual institutions, nine patients (52.9%) had no complications. In contrast, of 83 patients in whom the serum albumin level measured by both methods was lower than 3.8 g/dL, 16 patients (19.3%) had no complications. There was a significant difference in the incidence of complications ($P < 0.01$; data not shown).

The percentage of oxidized albumin in the total serum albumin level measured by immunoassay was plotted against the serum albumin levels measured by immunoassay (Figure 4). The oxidized albumin percentage increased as the serum albumin level decreased ($P < 0.001$). Plotting of the oxidized albumin percentage versus the Child class (Figure 5) demonstrated that oxidized

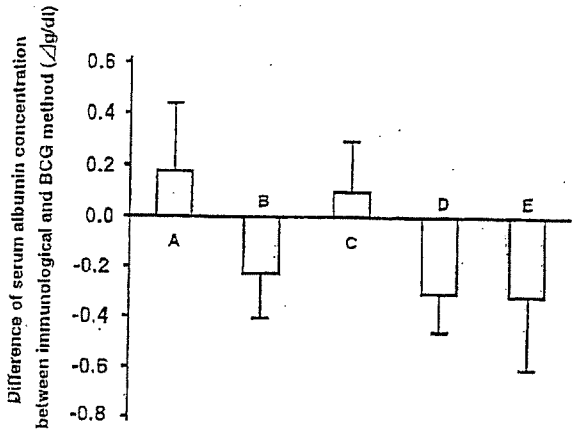

Fig. 3. Comparison of serum albumin levels in cirrhotic patients measured by the BCG method at five institutions. The serum samples were 48 samples whose serum albumin levels were 3.0 to 3.5 g/dL when measured by immunoassay at the contract laboratory. There were no significant differences in mean values across institutions. Differences (Δ g/dL) between values measured by the BCG method at individual institutions and those measured by immunoassay at the contract laboratory were calculated, and the mean ± standard deviation was plotted ($P < 0.05$). Site A ($n = 8$) versus site B ($n = 11$); site B versus site C ($n = 13$); site A versus site D ($n = 13$); site A versus site E ($n = 3$); site C ($n = 13$) versus site D ($n = 13$); site C versus site E ($n = 3$). At some institutions, mean serum albumin levels by the BCG method were 0.1 to 0.2 g/dL higher or 0.2 to 0.4 g/dL lower than those measured by immunoassay. BCG, bromcresol green (3,3′,5,5′-tetra-bromo-*m*-cresolsulfonphthalein).

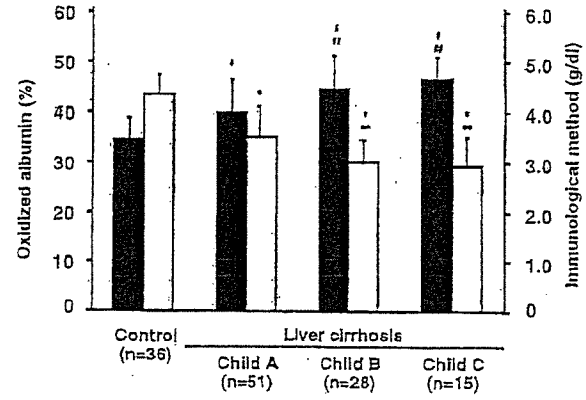

Fig. 5. Oxidized albumin percentage (solid bars) and serum albumin level (open bars) measured by immunoassay and classified by severity (Child's classification) in 94 cirrhotic patients. The oxidized albumin percentage by Child class increased with progression of the disease. Vertical bars show the standard deviation. Oxidized albumin percentage: control versus liver cirrhosis (Child A, B, and C), $\#P < 0.05$; Child A versus Child B and C, $\#\#P < 0.05$. Albumin level: control versus liver cirrhosis (Child A, Bs and C), $*P < 0.05$; Child A versus Child B and C, $**P < 0.05$.

albumin percentage increases with a decrease in total albumin concentration (Child A versus B, $P < 0.001$; Child A versus C, $P < 0.001$).

Patients were assigned to one of two groups: one group in which the difference in the measured serum albumin level between the BCP method and immunoassay was 0.3 g/dL or higher and another group in which it was lower than 0.3 g/dL. The oxidized albumin percentage was higher in the group with 0.3 g/dL or higher (data not shown). This suggested that albumin determination by the BCP method is affected by the oxidized albumin percentage. In cirrhotic patients, the reduced albumin percentage was more markedly decreased than the oxidized albumin percentage (data not shown). This is one reason the oxidized albumin percentage was increased.

Figure 6 shows the relation between the serum albumin level measured by immunoassay and the glycoalbumin percentage in 77 patients with liver cirrhosis but without diabetes, and Figure 7 shows the glycoalbumin percentage by Child class. Like the oxidized albumin percentage, the glycoalbumin percentage increased with a decrease in the serum albumin level measured by immunoassay ($P < 0.001$) and increased with the severity of the disease (Child A versus B, $P < 0.001$; Child A versus C, $P < 0.001$). In cirrhotic patients with diabetes, the mean glycoalbumin percentage

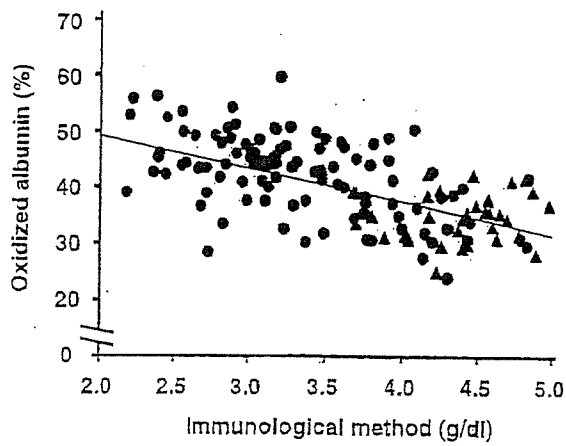

Fig. 4. Correlation between serum albumin levels measured by immunoassay and the oxidized albumin percentage method. In cirrhotic patients (circles; $n = 103$) and healthy controls (triangles; $n = 36$), the oxidized albumin percentage increased with a decrease in serum albumin level. Solid line, regression equation: $y = -0.06x + 0.61$ ($R^2 = 0.380$).

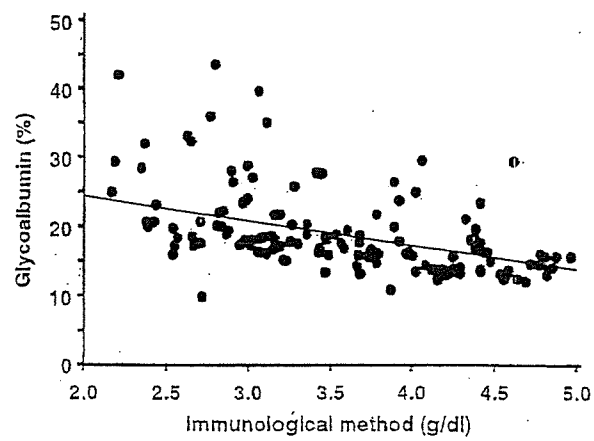

Fig. 6. Correlation between serum albumin level measured by immunoassay and the glycoalbumin percentage. In 78 cirrhotic patients without diabetes (solid circles), the glycoalbumin percentage increased as the serum albumin level decreased. Solid line, regression equation: $y = -3.56x + 31.52$ ($R^2 = 0.201$).