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KEYWORDS: -glutamyltransferase, hepatocellular carcinoma, ultracentrifugation, Affi-Gel Blue chromatography, Con A-Sepharose chromatography

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ISOLATION, CHARACTERIZATION AND CLINICAL EVALUATION OF THE \( \gamma \)-GLUTAMYLTRANSFERASE ASSOCIATED WITH HEPATOCELLULAR CARCINOMA

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Abstract. Sera from 24 patients with hepatocellular carcinoma (HCC), 30 patients with hepatobiliary diseases other than HCC and 5 normal subjects were analyzed for \( \gamma \)-glutamyltransferase (GGT) isozymes. In ultracentrifugation, GGT I' was recovered in the non-lipoprotein fraction (the residue), together with GGTs I'', I', I and X. GGT's III to IX were recovered in lipoprotein fractions. GGT's in the lipoprotein fractions were removed beforehand by Affi-Gel Blue chromatography, leaving GGTs I', I'', I', I and X in the non-bound fraction, which was subjected to Con A-Sepharose chromatography. From the double affinity chromatography (DAC), GGT's I' and II' were recovered in the unbound fraction, and GGT's I, I'', II' and X in the bound fraction. GGT activities in the unbound fractions of sera from HCC patients were generally higher than those from patients with other benign hepatobiliary diseases. When the GGT activity of the unbound fraction in DAC was expressed as a percent of the sum of the unbound and bound activities \((U/(U+B))\) and 22 % was set as the lower limit of positive values, 54 % of the HCC cases had positive values, while none of the patients with hepatobiliary diseases other than HCC had positive values. The \(U/(U+B)\) ratio of GGT in DAC appears to be a clinically useful test for screening HCC.

Key words: \( \gamma \)-glutamyltransferase, hepatocellular carcinoma, ultracentrifugation, Affi-Gel Blue chromatography, Con A-Sepharose chromatography.

\( \gamma \)-Glutamyltransferase (GGT, EC 2.3.2.2) is known as a marker of hepatocellular carcinoma (HCC) and preneoplastic lesions in experimental animals (1, 2). However, serum and tissue levels of GGT in humans do not serve as markers of HCC (3, 4). Usually, hepatobiliary obstruction or alcoholic liver injury causes more pronounced elevations in GGT activity. Köttgen was the first to make observations on the GGT-concanavalin A (Con A) interaction; the serum GGT from alcoholic liver injury patients was nonreactive, the GGT from normal controls reactive and the GGT from HCC patients partially reactive with Con A (5). GGT isozymes have been separated by polyacrylamide gradient (4-30 %) gel slab electro-

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phoresis (PAGGE) into 13 (Kojima et al. (6)) or 12 (Sawabu et al. (7, 8)) bands. Bands I’, I” and II’ (Kojima et al. (6)) or I’, II and II’ (Sawabu et al. (8)) have been regarded as hepatoma-associated GGT. Kojima et al. further demonstrated that GGT I’ and a part of GGT II’ were nonreactive with Con A, whereas GGT I, a ubiquitous isozyme, was reactive with Con A (9).

Since most of the serum GGT appears to be associated with lipoproteins (10) and some lipoproteins are reactive with Con A (11), the direct application of serum to Con A-mediated separation, as performed by Köttgen, can not be rationalized for the separation of different GGT molecules per se. Lipoprotein patterns themselves also change markedly in hepatobiliary diseases (12). In this study, the removal of lipoprotein-associated GGT from serum before its fractionation with Con A was attempted with Affi-Gel Blue in order to establish a system for fractionation of HCC-associated GGT. The results were evaluated from a clinical point of view.

MATERIALS AND METHODS

Materials. Serum was obtained from 54 patients with liver diseases, including 24 patients with HCC (14 cases were histologically diagnosed), 5 with acute hepatitis, 3 with chronic hepatitis, 3 with alcoholic liver injury, 3 with primary biliary cirrhosis (PBC), 2 with intrahepatic cholestasis, 3 with extrahepatic biliary obstruction (carcinoma of pancreas head) and 11 with cirrhosis, and from 5 healthy subjects. The serum samples were stored frozen at -20°C and analyzed within three months without noticeable changes in the results. Plasma lipoprotein fractions separated by ultracentrifugation were stored at 4°C and analyzed within a few days.

Electrophoresis. GGT isozymes were separated by PAGGE with a Model GE-4 electrophoretic apparatus (Pharmacia Fine Chemicals, Uppsala), using polyacrylamide gradient gel slabs PAA 4/30 (Pharmacia Fine Chemicals, Uppsala) and a buffer system of 3mM Tris-50mM glycine, pH 8.5. Electrophoresis was run for 24h at 125V while circulating tap water to cool the electrophoretic chamber. GGT on the gel plate was detected by a color reaction with Fast Garnet GBC (Sigma Chem. Co., Saint Louis) after incubation of the gel for 2h at 37°C in a reaction mixture containing N-γ-L-glutamyl-α-naphthylamide (Sigma Chem. Co., Saint Louis) as a substrate and glycglycine as a receptor according to a slight modification of the method of Sawabu et al. (7). The reaction time for color development was prolonged to 2h at 4°C in the present study. The GGT isozymes were numbered according to the system of Kojima and others (6).

Ultracentrifugation. Plasma samples were fractionated into lipoprotein classes (VLDL, very low density lipoproteins (d<1.006g/ml); LDL, low density lipoproteins (d = 1.006-1.063g/ml); HDL, high density lipoproteins (d = 1.063-1.21g/ml) and the residue) by preparative ultracentrifugation according to the method of Yasugi and Honma (13). These lipoprotein fractions were analyzed for GGT activity, and their isozymes were separated on PAGGE after dialysis against the electrode buffer. Lipoproteins for PAGGE were pre-stained with Sudan Black B (Merck, Darmstadt).

Immunoelectrophoresis (IEP). IEP was performed on 1% agarose (Agarose A, Pharmacia Fine Chemicals, Uppsala) gel prepared in Barbital Buffer-A (μ = 0.05 and pH 8.6; Daiichi Pure Chemical, Tokyo), which was also used as the electrode buffer. Electrophoresis was run for 1h at 1.5mA/cm, and immunodiffusion was performed for 24h at 4°C with rabbit antisera.
to human $\alpha_1$ and $\beta$ lipoproteins (Behring Institute, Marburg). GGT activity was detected as described above for PAGGE of GGT except for the reduction of time for incubation and color reaction to 1 h. Antigen-antibody precipitates were detected by transillumination.

**Affinity column chromatography.** Column chromatograms were run at room temperature (25 ± 3°C) with serum samples dialyzed against respective starting buffers. For Affi-Gel Blue column chromatography, 0.4 ml of serum was applied to an Affi-Gel Blue (Bio-Rad, California) affinity column (1.5 x 3.4 cm) and eluted with 18 ml of 0.02 M sodium phosphate buffer, pH 7.1 (Fraction I), 18 ml of 1.4 M NaCl in the same buffer (Fraction II) and 18 ml of 6 M urea also in the same buffer (Fraction III). Each fraction was concentrated with Minicon B-15 (Amicon Corp., Lexington) to the same volume as the applied sample and analyzed for GGT isozymes and lipoproteins by PAGGE. For Con A-Sepharose column chromatography, 0.4 ml of serum was applied to a Con A-Sepharose (Pharmacia Fine Chemicals, Uppsala) column (1.6 x 7.5 cm), and the column was washed with 150 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM MgCl$_2$, 1 mM MnCl$_2$, 1 mM CaCl$_2$, 0.5 M NaCl and 4 mM NaN$_3$. After collecting fractions of the starting buffer wash, GGT was eluted with 150 ml of the same buffer containing 0.2 M $\alpha$-methyl-D-mannoside (Sigma Chemical Co., Saint Louis) in 5-ml fractions at a flow rate of 10 cm/h. The protein profile from the column was monitored at 280 nm. Fractions with peaks of GGT activity were combined and concentrated to the original sample volumes and analyzed for GGT isozymes by PAGGE.

A simplified double affinity chromatography (DAC) with Affi-Gel Blue and Con A-Sepharose was run as follows: A small column (0.8 x 4 cm) packed with 0.75 ml of Affi-Gel Blue, and connected at its lower end to a similar column packed with 2 ml of Con A-Sepharose (Fig. 1), was equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.5 M NaCl and 4 mM NaN$_3$. Fifty $\mu$l of serum (GGT activities, 4-1333 U/l) was applied onto the upper column and washed with 15 ml of the equilibrating buffer to collect the unbound fraction. The upper column was removed and the lower one was eluted with 15 ml of 0.2 M $\alpha$-methyl-D-mannoside dissolved in

![Fig. 1. Schematic illustration of DAC and protein profiles showing the unbound and bound fractions. The arrow above the chromatogram indicates the removal of the top column and the change of buffer. U, unbound fraction, and B, bound fraction.](image)
the same buffer to collect the bound fraction. The two fractions were concentrated to the volume of the original sample and analyzed for GGT activity and its isozymes by PAGGE. GGT activity was measured spectrophotometrically by the method of Orlowski and Meister (14), using L-\(\gamma\)-glutamyl-p-nitroanilide as a substrate and glycylglycine as a receptor (\(\gamma\)-GT P Test-Pack AK, Sankyo Co., Tokyo). The percent activity of unbound GGT in DAC was calculated by the following formula:

\[
\text{unbound GGT activity (\%)} = \frac{U}{U + B} \times 100
\]

where U stands for GGT activity of the unbound fraction from DAC and B for GGT activity of the bound fraction. Coefficients of variation (\%) for \(U/(U + B)\) ratios determined in samples with GGT activities which ranged from 6 to 378 U/l were less than 18\% in samples with GGT activities above 42 U/l and greater than 18\% in those below 42 U/l, indicating that the present assay is fairly reproducible for samples with GGT activities above 42 U/l. The lower detection limit of the present assay was 0.35 U/l. Low U GGT values less than 0.35 were recorded conveniently as 0 for calculation of \(U/(U + B)\) ratios.

**Calculation of diagnostic indices.** Diagnostic indices were calculated as follows according to Davies and Gochman (15): The diagnostic sensitivity was derived using the number of patients with HCC having levels of \(U/(U + B)\) above the benign range as a percent of the total number with that disease. The specificity was calculated from the number of patients without HCC and normal subjects having levels of \(U/(U + B)\) within the benign range as a percent of all such patients and normal subjects. The efficiency of the test was obtained from the sum of the patients with true-positive and true-negative results as a percent of all the patients tested. The positive predictive value (true positives as a percent of all positives) and the negative predictive value (true negatives as a percent of all negatives) were also calculated.

**RESULTS**

Zymograms of GGT isozyme and lipoprotein profiles separated by PAGGE of plasma samples obtained from one case each of HCC and of alcoholic liver injury are shown in Figs 2a and b, respectively. GGT IX corresponded in mobility to LDL; the GGT band migrated between GGTs IX and X corresponded to VLDL, and GGTs III to VIII corresponded to HDL. The GGT bands from III to IX and one between IX and X are referred to as lipoprotein-associated GGT. In the case of alcoholic liver injury, GGT activity was mainly in the HDL fraction. The GGT in the residue of plasma samples of alcoholic liver injury patients was separated into GGT I and GGT X (high molecular lipid-protein complex). The residue GGT in plasma samples of HCC patients was separated into GGTs I, I', I'' and X, which were referred to as lipoprotein-unassociated GGT. The association of serum GGT activity with lipoproteins was also demonstrated by IEP with antisera to human \(\alpha_1\) and \(\beta\) lipoproteins (Fig. 3). GGT activity was revealed on the lines of immunoprecipitates for \(\alpha_1\) and \(\beta\) lipoproteins.

The above results indicate that the GGT isozyme pattern per se can not be obtained if serum is directly analyzed by electrophoresis. Either lipoproteins and GGT should be separated or lipoprotein-associated GGT should be removed be-
Fig. 2. GGT and lipoprotein bands in PAGGE. The horizontal arrow indicates the direction of electrophoresis. a, HCC (Case NT), and b, alcoholic liver injury (Case KA). The upper plates were stained for GGT activity and the lower plates for lipoproteins. GGT isozymes are numbered from I to X according to the system of Kojima and others (6) and are shown by vertical arrows.

Fig. 3. IEP. Immunoprecipitates were visualized by transillumination (left panel) and also stained for GGT (right panel). AHα_1LP, antiserum to human α_1 lipoprotein; AHS, antiserum to human serum, and AHβLP, antiserum to human β lipoprotein. Other symbols, see the legend to Fig. 2.

fore further analysis by electrophoresis. Since the GGT isozymes associated with HCC (GGTs I', I" and II') were recovered in the residue, attempts were made to remove lipoprotein-associated GGT from the serum by passing the serum through an Affi-Gel Blue column. With serum from a case of HCC, GGTs I, I', II' and X were recovered in Fraction I, and GGTs I" and II' in Fraction II. When serum from a case of PBC was used, the GGTs III to IX, which were recovered in lipoprotein fractions in ultracentrifugation, were not present in Fractions I and II, and were eluted with 6 M urea into Fraction III. The results are shown in Fig. 4.
Fig. 4. Gels stained for GGT activity after PAGE of sera and fractions of the sera obtained by Affi-Gel Blue chromatography. HCC, Case NT, and PBC, Case HY. Fr. I, Fraction I; Fr. II, Fraction II, and Fr. III, Fraction III. Other symbols, see the legend to Fig. 2.

When sera from a patient with HCC and a patient with alcoholic liver injury were analyzed by Con A-Sepharose column chromatography, the serum from the HCC patient gave three peaks of GGT activity corresponding to the unbound (pass-through fraction), loosely bound (subsequent unbound fractions) and bound fractions (eluate with α-methyl-D-mannoside), while the serum from the alcoholic liver injury patient gave two peaks, the unbound and bound fractions (Figs. 5 a and b). In the case of HCC, the unbound and loosely bound fractions contained GGTs I' and II' (faint in the photograph of the loosely bound fraction) and the bound fraction gave GGTs I, I'' and II'. In the case of alcoholic liver injury, GGT VI was recovered as a weak but major band in the unbound fraction, and GGTs I, III to IX and X were recovered in the bound fraction. In a case of alcoholic liver injury, the GGT activity was recovered mostly in the bound fraction (89%), while the GGT activity in HCC was recovered less in the bound fraction (65%).

The GGT isozyme patterns in Con A-Sepharose chromatography were considered to be simplified if Fraction I from the Affi-Gel Blue chromatography was applied to a Con A-Sepharose column, because the lipoprotein-associated GGT was then removed from Fraction I. This procedure was done either by serial application of separated Fraction I or by a one-step DAC with virtually identical results by either method. The bound and unbound fractions obtained by DAC of sera from patients with liver diseases were analyzed by PAGE and the results of representative cases are presented in Fig. 6. In cases of HCC the unbound fractions gave bands of GGT I' and faint bands of II', and the bound fraction gave intense bands of GGT I and faint bands of I'', II' and X (hardly visible in
Fig. 5. GGT activities in fractions separated by Con A-Sepharose chromatography and their zymograms by PAGGE (inset). a, HCC serum (Case NT), and b, alcoholic liver injury serum (Case SS). ———, protein; and ●●●●●●, GGT. O, original serum; U, unbound fraction; L, loosely bound fraction, and B, bound fraction. The arrow above the chromatogram, starting position of 0.2 M α-methyl-D-mannoside. Other symbols, see the legend to Fig. 2.
Fig. 6. PAGE patterns of GGT in sera from patients with liver diseases and in fractions of the sera separated by DAC. AH, acute hepatitis, and Alco, alcoholic liver injury. PBC, Case KK. Other abbreviations, see legends to Figs. 1, 2 and 5.

In cases with liver diseases other than HCC, the unbound fractions gave no-detectable bands except for a weak band of $I'$ in a case of PBC (Case KK in Table 1), while the bound fractions gave GGTs I and X. GGTs III to IX were not detected in either of the two fractions in the cases studied. Thus, the unbound fractions in cases of liver diseases without HCC had no demonstrable GGT $I'$. In sera from normal subjects, the unbound fractions had no visible GGT bands, but the bound fractions had GGT I and faint GGT X (not shown).

GGT activities of original sera and of the unbound and bound fractions obtained by DAC and percent of unbound GGT activity of all the cases examined are listed in Table 1. Higher GGT activities in the unbound fraction were found in patients with HCC than in those with other liver diseases. Correlation coefficients ($r$) between original and unbound GGT activities were 0.36 for HCC, 0.69 for other liver diseases and 0.21 for all the cases. On the other hand, the GGT activities in the bound fraction were related to those of the original serum (for
## Hepatoma Associated GGT

### Table 1. GGT activities of original serum and of unbound and bound DAC fractions, and the U/(U+B) ratio

<table>
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U*, unbound fraction from DAC; B**, bound fraction from DAC; Nor, normal subject; AH, acute hepatitis; CH, chronic hepatitis; Alco, alcoholic liver injury; IC, intrahepatic cholestasis; EBO, extrahepatic biliary obstruction, and LC, liver cirrhosis.
HCC, \( r = 0.51 \) and for other liver diseases, \( r = 0.91 \). When the activities of the unbound fraction were expressed as a percent of the sum of the unbound and bound activities (\( U/(U+B) \)), the difference between HCC and other liver diseases became apparent. The percent activity of unbound GGT was 0, or below 0.4 (the lower detection limit) in normal subjects and 0 to 21 in liver diseases without HCC. Thus, the benign range was set from 0 to 21 percent. The percents of unbound GGT activity of all the cases are reiterated in Fig. 7. These values were used for calculation of diagnostic indices. Thirteen out of 24 patients with HCC had levels of \( U/(U+B) \) greater than 22% (sensitivity, 54%), whereas no healthy subjects and none of the 30 patients with liver diseases other than HCC had positive values\(^1\). The specificity was calculated to be 100% since none of the cases without HCC had positive values. The efficiency of the test was also high (81%) because of a small number of false-positive and false-negative results. The predictive value of a positive test was 100% as may be expected from the specificity of 100%. The predictive value of a negative test was slightly low (76%) due to 11 false negative results in HCC.

When the levels of unbound GGT were compared with the presence of GGT I’ in PAGGE, all of the HCC cases with unbound GGT activities greater than 5 \( U/1 \) had GGT I’, whereas only one case of PBC with unbound GGT activity of 6 \( U/1 \) showed trace activity of GGT I’ (Fig. 6), indicating that the increased GGT activity in the unbound fraction is largely due to the increase in GGT I’ activity. The increases in GGT activity of the unbound fraction and in the percent activity

\(^1\) In normal subjects except Case AW, \( U/(U+B) \) ratios would range up to 15%, as the U values less than 0.35 U/1 were recorded as 0 (see Materials and Methods).
of unbound GGT in HCC were unrelated to the size of the tumor mass in HCC patients, the level of serum α-fetoprotein and the histological grading determined according to Edmondson and Steiner (16) (Table 2).

**DISCUSSION**

The GGT isozymes I', I" and II' which are frequently detected by PAGGE in sera from patients with HCC were present in the residue fraction in ultracentrifugation (17), unlike the other GGT isozymes, which were associated with lipoproteins, as previously demonstrated by Watanabe et al. (10). HCC-associated GGT was partially purified by removing the lipoprotein-associated GGT simply by adsorption of lipoproteins (18) to the Affi-Gel Blue column, leaving GGTs I, I', I", II' and X in the residue or in the pass-through fraction. The association of GGT with lipoproteins was further substantiated in the present study by IEP with anti-human-α, and β lipoprotein sera. Since a part of the HCC-associated GGT was shown by Kojima and others (9) to be nonreactive with Con A, the HCC-associated
GGT was separated by Con A-Sepharose chromatography. This technique has some advantages over the PAGGE used by Sawabu (7) in that the results can be expressed quantitatively and the separation of GGT I' from GGT I is much clearer. The major component in the final unbound fraction obtained by DAC in the present study was GGT I'. Thus, the GGT activity in the unbound fraction of DAC is a new parameter differing from the respective GGT isozymes detected by PAGGE. Although higher GGT activities in the unbound fraction were found in liver diseases other than HCC when serum GGT activities were high (r = 0.69, p < 0.001), the percent contribution of this activity to the total activity was negligible (less than 2%). Furthermore, the correlation coefficients for HCC cases and all of the cases were 0.36 (p > 0.05) and 0.21 (p > 0.05), respectively, indicating that the activities of the unbound fraction are independent of the original GGT activities as a whole. In this connection, the clinical significance of the new parameter was tested on sera of various liver diseases. The ratio of unbound to the sum of the unbound and bound GGT activity (U/(U + B)) best discriminated the HCC patients from the patients with other liver diseases when 21% was set as the upper limit of non-HCC values. The sensitivity was 54% without false positive results. Therefore, the test is quite specific to HCC and efficient for screening HCC, if values greater than 22% are taken as positive. Even α-fetoprotein, which is the most specific tumor marker of HCC, had a sensitivity of 75% with a specificity of 98%, when the upper limit of the α-fetoprotein for the benign range was set at 500 ng/ml.

The incidence of positive GGTs I', I'' or II' in HCC is reported to be 60% by Kojima et al. (6) and 55% by Sawabu et al. (8). These values agree well with the positive incidence of U/(U + B) in the present study. Despite such a high incidence and specificity of HCC-associated GGT in HCC, its clinical application is limited, mainly because the discrimination of the most intense band of HCC GGT I' from the ubiquitous band of GGT I is difficult due to their small difference in electrophoretic mobility on the polyacrylamide gel. In the present study, this problem was eliminated by separating GGT I' from GGT I on the basis of their difference in affinity for Con A-Sepharose. The present DAC analysis of HCC-associated GGT may prove to be a clinically useful tool in the diagnosis of HCC because of its simplicity and high specificity to HCC.

It is of some interest that a case of PBC showed a relatively high value of U/(U + B) and a faint but discernible GGT I' band. Four cases (1 of metastatic liver cancer, 1 of chronic hepatitis and 2 of alcoholic liver injury) among 279 non-HCC liver disease cases were reported by others to have GGT I'. (8) Relatively high values of U/(U + B) were also noted in cases of acute hepatitis in this study.

* Among the α-fetoprotein levels reported by Yachi and Suga (19), those in liver diseases (HCC, acute hepatitis, chronic hepatitis and cirrhosis) were used for calculation of the indices according to Davies and Gochman (15).
Hepatoma Associated GGT

(2 Table 1 and Fig. 7). Undifferentiated phenotypical expression in non-malignant hepatic injury is well documented (20, 21) for key glycolytic and gluconeogenic enzymes and α-fetoprotein (22, 23). Undifferentiated hepatic enzyme patterns were also reported in experimental biliary obstruction (24). Under these conditions, fetal or prototype liver enzyme and protein levels increase in the injured liver and serum. It is, therefore, not surprising that GGT I′ appears in acute hepatitis and PBC as an undifferentiated phenotypical gene expression due to liver injury.

Another interesting fact is that HCC can be differentiated from other benign liver diseases solely on the difference in affinity of GGT for Con A. Sugar chains of HCC GGT in rats have been shown to have a bisecting N-acetylglucosamine in the bianennary asparagine type oligosaccharide and also tri- and tetra-antennary structures (25). These sugar chains have no affinity for Con A (26, 27). Similar oligosaccharides appear to be present in human HCC GGT, because the GGT which was characteristic of HCC was found to be unreactive with Con A in the present study. The bisecting N-acetylglucosamine linked to the β-linked mannose residue was shown by Commings and Kornfeld (28) and Yamashita (29) to be specifically reactive with the erythroagglutinating phytohemagglutinin form of Phaseolus vulgaris agglutinin (E-PHA). The Con A-nonreactive GGT in human HCC has a higher affinity for E-PHA than that in other benign liver diseases or the Con A-reactive GGT in HCC (30). This result indicates the presence of further heterogeneity of Con A-nonreactive GGT with respect to the affinity for E-PHA. Generalization of this observation to other tumor markers has to be made with caution since an opposite relationship exists with α-fetoprotein (31).

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REFERENCES


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