Abnormal expression of hepatoma-derived γ-glutamyltransferase subtyping and its early alteration for carcinogenesis of hepatocytes

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BACKGROUND; Although the hepatoma-specific band of gamma-glutamyltransferase (GGT) is a highly sensitive marker in diagnosis of hepatocellular carcinoma, the kinetic expression and the early alterations of GGT in the development of hepatoma remain unclear. In this study, we investigated the expression and the alterations of GGT multiple molecular forms in hepatotumorigenesis.

METHODS; The expression of GGT in a chemically induced hepatocarcinogenesis model was examined by giving 0.05% of 2-fluorenylacetamide in diet for 12 weeks. The expression levels of total RNA and GGT, and the changes of liver pathology, GGT multiple molecular forms and sugar-chain heterogeneity were investigated at the different stages of rat hepatoma development.

RESULTS; Pathological examination and biochemical analysis found that liver GGT was over-expressed and secreted into blood during carcinogenesis. Serum total GGT and liver GGT specific activities (IU/g) including soluble and membrane-combined GGT were significantly higher (P < 0.05) in experimental groups than those in control group, respectively. A highly positive correlation was found between total GGT activities and total RNA levels (r = 0.90, P < 0.05) of the liver. Both were higher six weeks later than before. Con A-non-reactive-GGT was increased consistently during the development of rat hepatoma. GGT multiple molecular forms in the liver and sera of experimental rats showed that fetal liver-type GGT bands were associated with the development of hepatoma.

CONCLUSIONS; Fetal liver-type GGT in sera and the liver of rats is closely related to hepatotumorigenesis. It can be used as a sensitive enzymatic marker for the early diagnosis of liver cancer.

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KEY WORDS; gamma-glutamyltransferase; multiple molecular forms; rat hepatoma; expression; sugar-chain heterogeneity

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and rapidly fatal malignancies worldwide, and has been ranked as the second cancer killer in China since the 1990s, particularly in the eastern and southern areas, including the inshore area of the Yangtze River. Major risk factors for HCC in these areas are exposure to aflatoxin B1 (AFB1) and infection by hepatitis viruses. The prognosis of HCC is poor and early detection is of utmost importance. Its treatment options are severely limited by the presence of metastasis. Although serum α-fetoprotein (AFP) is a useful tumor marker for the detection and monitoring of HCC development, the false-negative rate with AFP level alone may be as high as 40% for patients with small size HCC. However, if hepatocyte-specific mRNAs are detected in the circulation, it is possible to infer the presence of circulating, presumably malignant, liver cells and to predict the likelihood of haematogenous metastasis.

Enzyme gamma-glutamyltransferase (GGT) frequently overexpressed in cancer cells and tissues has been significantly used as a cancer marker. It is a membrane-bound ectoenzyme that catalyzes the degradation of glutathione and other gamma-glutamyl compounds by hydrolysis of gamma-glutamyl moiety or by its transfer to a suitable acceptor. Serum GGT activity as a sensitive marker of hepatobiliary disorders, exhibits a tissue-specific expression under various physiological and pathological conditions. The activity of GGT is the highest in
GGT expression in hepatoma

...embryo liver and decreases rapidly to the lowest level after birth, and it is a widely distributed enzyme that has been extensively studied in relation to hepatocarcinogenesis. GGT is a heterodimeric glycoprotein, and several experiments have demonstrated that these increases are often associated with structural changes in the sugar-chains of the enzyme, as evidenced by a variation in the pattern of GGT isofoms in serum. Previous investigations suggested that the evaluations of GGT multiple forms might improve the specificity of GGT measurement, indicating that there might be a correlation between specific patterns and different disease states.

GGT is mainly present on hepatocyte cytoplasm and epithelial cells of the bile duct. Total GGT activities make differential diagnosis difficult, because GGT is over-expressed in hepatobiliary diseases. Recently, analysis of GGT isoforms has shown that the incidence of GGT hepatoma-specific band (HSB) is 87% in patients with HCC. However, the kinetic alterations and expression of GGT at the early stage of hepatoma development remain unclear. In the present study, expression and kinetic alterations of GGT and its sugar-chain heterogeneity were observed in rats with hepatoma induced with 2-fluorenylacetamid (2-FAA) at different stages of HCC development.

Methods

Hepatoma models

Forty-two closed colony male Wistar rats weighing 140–180 g obtained from the Experimental Animal Center (Shanghai, China) were divided randomly into seven groups, one normal control group (n=6) and six experimental groups (n=6 for each group). All rats were housed under bio-clean conditions at a temperature-controlled (22 ± 2 °C) environment with a 12-hour light/dark cycle and 55% humidity, and the control group was given basal diet and the diet contained 0.05% 2-FAA (Sigma, USA). The rats were then monitored daily for survival and weight loss, while recording their clinical signs. They were sacrificed at different time. One group was killed every two weeks, and surgical procedures were conducted under deep ether anaesthesia. Blood was drawn from the rats, their livers were collected, and histological examination was performed with hematoxylin and eosin (HE) staining. The procedures were conducted in accordance with the guidelines for experimental animals approved by the Animal Care and Use Committee of Nantong University, China.

Purification of liver GGT

Fresh adult rat livers and fetal rat (18 days) livers were washed with 0.9% NaCl solution, dried with filter paper, and cut into pieces. The rat liver was added with 5 ml homogenate solution (pH8.6, 0.1 mol/L Tris-HCl buffer I) to extract soluble GGT or added with 5 ml homogenate solution including 0.5% Triton-X 100 (buffer II) to extract total GGT, homogenized at 4 °C at 12 000 r/min in a YQ-3 type homogenizer (Shanghai, China), stopped 5 minutes in every 30 seconds for 5 times, then centrifuged at 15 000 r/min for 45 minutes at 4 °C. The supernatants were taken and stored at -20 °C for GGT activity, electrophoresis, and affinity analysis.

Isolation of liver total RNA

Total RNAs were isolated from rat liver homogenates with RNAzole reagent (Gibco BRL, USA) and purified according to the described protocol. The total RNA concentrations of rat livers were assessed by measurement of optical density at 260 nm in an ultraviolet spectrophotometer (Shimadzu UV-2401, Japan).

Detection of GGT activities and multiple forms

Analyses of total GGT activity or GGT fractions were performed within one day of blood collection. The total activities (IU/L) of serum and liver GGT were measured by a p-nitroanilide direct assay. The protein concentrations of the supernatants were detected by a Folin–phenol reagent assay. The specific activity (IU/g) of liver GGT was calculated according to the ratio of GGT activity (IU/L) to protein concentration (g/L). To compare with the alterations of GGT levels in sera of the experimental rats, the activities of alanine aminotransferase (ALT), adenosine deaminase (ADA) and glutathione S-transferase (GST) were simultaneously determined.

The modified separation technique for serum and liver GGT multiple molecular forms was used. Briefly, a vertical slab apparatus (Beijing, China) was loaded with polyacrylamide stage gel containing one layer of concentration gel of 4.0% on the top and three layers of separation gel of 7.7%, 11.5% and 15.0% in the ascending order. Then, serum or liver supernatant (20 μl) was mixed with an equal volume of 40% sucrose bromophenol blue and introduced into sample holes in the concentration gel. These samples were electrophoresized at 60 V until the sample migrated into the separation gel, and then, at a constant voltage of 200 V for 4 hours. Thereafter, the gel plate was taken out from the electrophoresis apparatus and wrapped with cellulose acetate sheets wetted through by GGT substrate solution and incubated at 37 °C for 45 minutes. Finally, the cellulose acetate sheets were immersed in a 50 ml solution containing 10% trichloroacetic acid and 25% glycerol, within two minutes, the red bands of GGT isoenzymes appeared on the cellulose acetate sheets.

Affinity analysis of rat liver GGT

GGT sugar-chain heterogeneity was detected by a mi-
column chromatography with concanavalin A (Canavalia
Ensiformis, Con-A, Sigma, USA)-Sepharose 4B affini-
ty.20,21 In brief, Con-A-Sepharose 4B gel was filled in-
to the micro-column (0.8 cm×8 cm) with natural sedi-
mentation and balanced with buffer I (0.1 mol/L Tris-
HCl buffer containing 1 mmol MgCl₂, 1 mmol CaCl₂,
0.1 mol NaCl, 1% Triton X-100, pH 8.0), the superna-
tant was added into the column, which was drawn,
and washed with 0.5 ml buffer I, and discarded. The first
part of GGT was washed with 3.0 ml buffer I and collect-
ed in one test tube, and the second part of GGT was col-
clected into the other test tube with 6.0 ml buffer II (in
buffer containing 0.2 mol α-methyl-D-galactoside). The
activities of the two parts of GGT were detected and the
percentage of GGT was calculated, respectively.

Statistical analysis
All rats were divided into seven groups. The results
were evaluated statistically by the Student's t test, the
chi-square test and the Fisher's exact probability test.
Probability values less than 0.05 were considered signifi-
cant.

Results

Histopathological findings
The pathological changes of rat livers are shown in Table
1. The rats were administered with 0.05% of 2-FAA. At
the end of the 2nd week, the appearance of the livers
turned to be grey-yellow and scabrous. Hepatocytes
showed granular degeneration at the early stage of liver
cancer, with normal liver tissue structure, a few hyper-
plastic oval cells, and initial nodule formation. At the end
of the 4th week, precancerous lesions appeared in half of
hepatic tissues. Histologically normal liver follicles existed
in most part of areas but hyperplastic small round cells or
oval cells or hyperplastic nodules in local areas. After six
weeks, the rat liver entered canceration stage, and the
structure of hepatic lobules was completely destroyed,
with necrosis and a large number of small oval cells and
cancer nests. Histological finding of the rat liver con-
formed diffuse patchy necrosis and highly differentiated
HCC.

Changes of liver total RNA and GGT levels
The levels of total RNA and hepatic GGT were ob-
served at the different stages during the induction of rat
hepatomas (Fig. 1). Both total RNA concentration and
GGT specific activities increased gradually during the ad-
ministration of 2-FAA. The activities of the rat liver and
serum GGT were significantly higher in the experimental
rats than those in controls. In the early stages of rat hepa-
toma development, GGT activities increased 5 to 10
times in the rat liver, and 4 to 8 times in rat sera com-
pared with those in the controls. The expression levels
of liver GGT in hepatoma rats approach to those in fetal
rat livers (Fig. 2). A positive correlation was noted be-
tween GGT specific activities and total RNA concentra-
tion (r=0.90, P<0.01) or between serum GGT ac-
tivities of the experimental rats and liver total RNA con-
centration (r=0.79, P<0.01). The kinetic alterations of
serum GGT average levels at different stages and the
comparison with other hepatic enzymes (ALT, ADA
and GST) are shown in Fig. 3. The activities of rat ser-
um ADA, GST and ALT were significantly higher than
those of GGT at the stage of hepatocyte degeneration,
and subsequently they decreased to normal, but in-
creased consistently six weeks later.

Changes of different-type GGT and its sugar-chain heterogeneity
The specific activity alterations of GGT of different
molecular form and its sugar-chain heterogeneity in rat
livers are shown in Table 2. The levels of hepatic soluble
and membrane-combined GGT increased markedly dur-
ing the course of liver cancer induction, especially in the
late phase. A significant difference was found between
the experimental groups and the control group (P<
GGT expression in hepatoma

Fig. 2. The kinetic alterations of liver GGT specific activities (IU/g wet liver, n = 6 for each group except for the 12th group) at different stages of rat liver cancer development and comparison with fetal rat liver GGT. FL: fetal rat liver; NC: normal control rat liver; GGT: gamma-glutamyltransferase.

Fig. 3. The kinetic alterations of serum GGT average levels (IU/L, n = 6 for each group except for the 12th group) at different stages of rat liver cancer development and comparison with three hepatic enzymes. NC: normal control; GGT: gamma-glutamyltransferase; ALT: alanine amino-transferase; ADA: adenosine deaminase; GST: glutathione S-transferase.

Table 2. Changes of different-type GGT and affinity analysis of GGT enzyme proteins at different stages of rat hepatoma development

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Liver GGT (IU/g)</th>
<th>Con A-affinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soluble-type</td>
<td>Membrane-type</td>
</tr>
<tr>
<td>Normal control</td>
<td>6</td>
<td>0.7±0.1</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>2nd week</td>
<td>6</td>
<td>3.8±2.8*</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>4th week</td>
<td>6</td>
<td>7.1±0.9**</td>
<td>2.3±1.3**</td>
</tr>
<tr>
<td>6th week</td>
<td>6</td>
<td>3.5±0.7**</td>
<td>2.5±1.3**</td>
</tr>
<tr>
<td>8th week</td>
<td>6</td>
<td>8.7±5.2**</td>
<td>2.4±1.5**</td>
</tr>
<tr>
<td>10th week</td>
<td>6</td>
<td>12.2±5.5**</td>
<td>3.8±1.3**</td>
</tr>
<tr>
<td>12th week</td>
<td>5</td>
<td>9.6±2.3**</td>
<td>6.2±4.4**</td>
</tr>
</tbody>
</table>

GGT-R-C: Con A-reactive-GGT; GGT-N-C: Con A-non-reactive-GGT. *: P < 0.05, **: P < 0.01, compared with the normal control.

Table 3. Electrophoresis patterns of liver (serum) GGT isoenzymes in the experimental rats, fetal liver and normal control

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>GGT isoenzyme bands on electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Normal control</td>
<td>6</td>
<td>0(0) *</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>2nd week</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4th week</td>
<td>6</td>
<td>3(3)</td>
</tr>
<tr>
<td>6th week</td>
<td>6</td>
<td>6(6)</td>
</tr>
<tr>
<td>8th week</td>
<td>6</td>
<td>6(6)</td>
</tr>
<tr>
<td>10th week</td>
<td>6</td>
<td>6(6)</td>
</tr>
<tr>
<td>12th week</td>
<td>5</td>
<td>5(5)</td>
</tr>
</tbody>
</table>

*: 0(0) expresses band numbers of liver GGT (serum GGT) isoenzymes; ++: or −: the low or high GGT levels on origin location of electrophoresis.

0.05). The activities of the two different molecular forms of GGT increased parallely, and the ratio of soluble GGT to total GGT was about 0.7. During the development of rat hepatomas, the liver GGT of the normal or hepatoma rats presented two forms, that is, Con A-reactive-GGT (GGT-R-C) and ConA-non-reactive-GGT (GGT-N-C), detected by Con A-sepharose 4B micro-column affinity chromatography. The GGT-N-C levels two weeks later were significantly higher (P < 0.05 or P < 0.01) in the experimental groups than in the control group, showing a tendency of persistent increasing.

Electrophoresis changes of rat serum and liver GGT

Different electrophoresis patterns of serum or liver GGT isoenzymes were found among control, fetal, and experimental rats by a vertical slab electrophoresis on polyacrylamide gradient gel. GGT isoenzymes were
separated into one to six bands from the sera and liver of these rats. The changes and electrophoresis patterns of the sera and liver GGT isoenzymes in the experimental rats with fetal or control rats were observed (Table 3). The fetal liver-type patterns of GGT isoenzymes were isoenzyme-I, II, III, IV, V, and VI. Only one band (isoenzyme-II) was found in the sera and livers of the controls; however, mixed six bands (isoenzyme-I, II, III, IV, V, and VI) were detected in the sera and livers of the experimental hepatoma rats. The data indicated that the patterns of GGT isoenzymes in the hepatoma rats were different from those in the normal rats, and that the fetal-type GGT isoenzymes were associated with hepatotumorigenesis and appeared in the early stage of hepatoma development.

Discussion

The prognosis of HCC is still poor at present. Exact diagnosis and proper treatment of HCC at early stages are required for a better prognosis. Serum GGT activity is considered a sensitive marker of hepatobiliary disorders. In the rat liver, the high GGT activity observed at the fetal stage decreases rapidly at birth, being barely detectable in the adult. GGT is often re-expressed in adult rat liver–bearing hepatocarcinogenesis and this is why it has long been regarded as an early marker of cellular dedifferentiation, signaling a reversion of the hepatocyte to a fetal phenotype. In rats with 2-FAA at early stage, their livers showed pathologically hyperplastic oval cells that were closely related to the development of hepatomas and the tendency of nodule formation. The oval cells not only had the cytotoxicity resistant to carcinogenesis and showed indiffident morphological shapes of hepatic cells, but also overexpressed GGT. In the present study, GGT was strikingly activated during the course of tumorigenesis in rat hepatoma models (Table 1), and was significantly increased at the degeneration (P < 0.05) and precancerous stages (P < 0.01) of hepatocytes. The increase of rat serum GGT activity was associated with an elevation of liver GGT activity. The data indicated that the expression of liver GGT is related to the development of rat hepatomas.

The alterations of GGT sugar-chain structure occurred during the development of hepatomas. Yamashita et al. compared the sugar-chains of hepatoma GGT and normal rat liver GGT, and found marked difference of the two kinds of GGT in enzymatic molecular composition. The sugar–chain of normal rat liver GGT was acidic while that of hepatoma GGT was 72% in acidity and 28% in alkaline. After canceration, the total saliva acid and sugar-chains of hepatoma GGT increased to 3–4 times in the hepatoma rats than in the controls. Normal liver GGT contained complete Gal1–4GlcNAc 1–outer sugar-chains, whereas hepatoma GGT showed incomplete sugar-chains, usually without galactose (Gal) outer sugar-chains. In this study, liver GGT-N-C concentration of the experimental rats consistently increased during the carcinogenesis of the rats induced with 2-FAA (Table 3). These findings suggest that both liver GGT levels and the sugar-chain heterogeneity of GGT molecule increase apparently in the process of carcinogenesis. Therefore, the detection of GGT-N-C concentration may be useful to hepatoma diagnosis.

The measurement of serum GGT activity is used as a routine laboratory test, which is generally accepted as the most sensitive marker of cholestasis, pancreatic disease, and enzymatic induction by alcohol and drugs. Total GGT activities make differential diagnosis difficult, because GGT is over-expressed in all hepatobiliary diseases. Serum GGT levels are affected by several other factors and a variety of clinical conditions, the measurement of serum total GGT is lack of specificity, and its value in the differential diagnosis is limited because of great overlapping. In the present study, the evaluations of fetal liver-type GGT fractions improved the specificity of the GGT measurement, indicating that there might be a correlation between specific patterns and different disease states and that the fetal liver activity of GGT is increased in carcinous hepatocytes and the fetal forms of GGT can be detected in sera of rats with liver malignancy by analysis of electrophoresis patterns.

GGT isoenzymes of human hepatoma, non-hepatoma tissues and serum in patients with HCC were analyzed in the previous studies and GGT of the liver supernatant and sera of HCC patients showed the hepatoma specific GGT band located on α1–region of protein electrophoresis, while the non-hepatoma tissues couldn’t show the band. These data suggested that hepatoma specific GGT band(s) be derived from hepatoma microsome. Also, the GGT activity could be found in the supernatant, granule parts of fetal liver, and experimental hepatomas. In this study, different multiple forms of rat livers or serum GGT were separated into 1 to 6 bands by a vertical slab electrophoresis using stage gradient polyacrylamide gel. Different electrophoresis patterns of serum or liver GGT were found in the control, fetal and experimental rats (Table 3). The analysis of multiple molecular forms of rat GGT showed five GGT bands (I, II, III, IV, V and VI) in the fetal rat livers, all six bands in sera and livers of the experimental hepatoma rats, and only one band (II) in the control rats. The fetal-type GGT appeared at the degeneration stage of rat hepatocytes, and the appearance of fetal liver-type GGT was slightly earlier in liver than in serum, showing its early diagnostic value for hepatomas. The recent albumin–GGT in cirrhosis with HCC would be a sensitive diagnostic marker of both advanced and small HCC-complicating liver cirrhosis.

In conclusion, the expression levels of GGT are...
significantly increased in the development of HCC, and the fetal liver–type isoenzymes could be detected in peripheral blood at the early stage of HCC. The circulating GGT isoenzymes could be a useful molecular marker for HCC diagnosis, especially in the early diagnosis of HCC. Further studies will explore the molecular mechanisms of reactivation of GGT in the development of HCC.

Competing interest
The author or one or more of the authors have received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of this article.

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Those who can take in necessary nutrition are healthier than those who eat more; similarly, a real scholar is not always the man who reads widely but the one who reads useful books.

— (Ancient Greece) Aristippus