

The analysis of γ -glutamyl transpeptidase gene in different type liver tissues

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Abstract

AIM: To probe the value of γ -glutamyl transpeptidase (GGT) messenger RNA in monitoring canceration of liver cells and for early diagnosis of hepatocellular carcinoma (HCC), by researching the types of GGT messenger RNA (GGTmRNA) in liver tissues and peripheral blood of different hepatopathy.

METHODS: The three types of GGTmRNA (A, B, C) in liver tissues and peripheral blood from the patients with HCC, noncancerous hepatopathy, hepatic benign tumor, secondary carcinoma of liver, and healthy persons were detected by reverse-transcription polymerase chain reaction (RT-PCR).

RESULTS: (1) In normal liver tissues, type A was predominantly found (100.00 %), type B was not found, type C was found occasionally (25.00 %). (2) The distribution of types of GGTmRNA in liver tissues with acute hepatitis, chronic hepatitis, cirrhosis, alcoholic hepatopathy was similar as in normal liver tissues ($P>0.05$), but type B was found in 3 of 18 patients with chronic hepatitis (16.67 %), and also in 3 of 11 patients with cirrhosis (27.27 %). (3) There was no significant difference of types of GGTmRNA between liver tissues with hepatic benign tumor, secondary carcinoma of liver and normal liver tissues ($P>0.05$). (4) Type B was predominant in cancerous tissues with HCC (87.5 %), the prevalence of type B in cancerous tissues was significantly higher than that in normal liver tissues (0/12) ($P<0.05$), but the prevalence of type A in cancerous tissues (46.88 %) was significantly lower than that in normal liver tissues (100.00 %) ($P<0.05$), and the prevalence of type C (6.25 %) in cancerous was the same as that in normal liver tissues (25.00 %) ($P>0.05$). In noncancerous tissues of livers with HCC, the main types were type A and type B, the prevalence of type A (85.71 %, 90.48 %) and type C (14.29 %, 9.52 %) in noncancerous tissues of liver with HCC was similar as that in normal liver tissues (A: 100.00 %; C: 25.00 %) ($P>0.05$), but the prevalence of type B (80.95 %, 76.19 %) in noncancerous tissues of livers with HCC was significantly higher than that in normal liver tissues (0/12) ($P<0.05$). (5) The prevalence of type B (37.5 %) in peripheral blood with HCC was higher than that in normal person (0/12) ($P<0.05$). In peripheral blood, type B was found in 4 of 11 cases of HCC with serum AFP negative.

CONCLUSION: The shift of types of GGTmRNA from A to B in liver tissues may be closely related to the development of HCC, and the analysis of GGT gene may provide a useful tool for early diagnosis of HCC.

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INTRODUCTION

HCC is one of common fatal malignant tumors^[1-5]. About 90 percent of tumors originated from liver are HCC^[6-10]. There are more than 250 000 patients of HCC in the world every year, 44.7 percent of these patients are Chinese^[11-15]. The pathogenic mechanism, early diagnosis, and efficient treatment of HCC are very important. Many studies concerning GGT in HCC have suggested that changes in hepatic GGT expression may be closely related to the development of HCC. It has been reported that HCC in humans expresses GGT enzymes with unique carbohydrate moieties compared with normal liver enzymes. The presence of the unique GGT isoform for HCC in patient sera was used as a marker for the diagnosis of HCC. However, the genomic changes in GGT relating to the development of HCC are not known, and genomic analysis of the specific GGT to HCC is also lacking. In this study, the specific primer sets for reverse-transcription polymerase chain reaction corresponding to the 5' -noncoding human GGTmRNA of fetal liver (type A), HepG2 cells (type B), and placenta (type C) were prepared. In order to clarify the type of GGTmRNA in human liver and the relationship between alterations in GGTmRNA expression and the development of HCC, we detected the types of GGTmRNA in normal liver tissues and diseased liver tissues with or without HCC.

MATERIALS AND METHODS

All cases were divided into 8 groups by type of diseases: (1) healthy control group 12 cases, male 9 cases, female 3 cases. (2) HCC group 32 cases, male 26 cases, female 6 cases, small sized HCC (size ≤ 3 cm) 8 cases, large sized HCC (size 3-10 cm) 16 cases, enormous sized HCC (size >10 cm) 8 cases. 11 HCC patients with serum alpha fetoprotein (AFP) negative, 21 HCC patients were treated with surgery. (3) Acute hepatitis group 15 cases, male 11 cases, female 4 cases, hepatitis A 4 cases, hepatitis B 3 cases, hepatitis E 5 cases, drug hepatitis 3 cases. (4) Chronic hepatitis group 18 cases, male 12 cases, female 6 cases, hepatitis B 14 cases, hepatitis C 4 cases. (5) Cirrhosis group 11 cases. The diagnosis of acute hepatitis, chronic hepatitis and cirrhosis meet the standard of National Academic Conference about infectious disease^[16]. (6) Alcoholic hepatopathy group 13 cases, male 13 cases. (7) Hepatic benign tumor group 10 cases, male 8 cases, female 2 cases, hepatic hemangioma 6 cases, hepatic cyst 4 cases. (8) Secondary carcinoma of liver 13 cases, male 8 cases, female 5 cases, primary carcinoma of stomach 4 cases, primary carcinoma of colon 4 cases, primary carcinoma of lung 2 cases, primary carcinoma of prostate 1 cases, primary carcinoma of ovary 2 cases.

Sample collection

Collection of liver tissues: Cancerous tissues, adjacent

paracancerous tissues (to the brim of carcinoma 3-5 cm) and distal cancerous tissues (to the brim of carcinoma >5 cm) were respectively obtained during surgery from 21 patients with HCC, and the cancerous tissues and noncancerous tissues were obtained by needle liver biopsy with help of ultrasound B from the other patients of HCC (11 cases). Liver tissues were obtained by needle liver biopsy in the other groups, non-tumor tissues were merely obtained in hepatic benign tumor and secondary carcinoma of liver. All the tissue specimens were immediately refrigerated by liquid nitrogen for 1 hour, and then were transferred to refrigerator at -80°C for future use.

Blood samples: peripheral blood was obtained from each person at morning before breakfast, anticoagulated by sodium citrate solution, and then peripheral blood mononuclear cells (PBMC) were separated from blood, total RNA was extracted from PBMC, cDNA was synthesized with reverse transcription, and then reserved in refrigerator at -80°C .

Total RNA extraction

Total RNA was extracted by using TRIzol (from TECH-LINE company) and the purity of RNA was tested with ultraviolet spectrophotometer of DAOJUN UR-2201.

Detection of the types of GGTmRNA by RT-PCR

cDNA was synthesized with RNA reverse transcriptase (M-MLV) and random primer (Oligodts). then cDNAs of GGTmRNA were amplified by PCR using polymerase and three different primer sets which were specific for the three GGTmRNA types. Nucleotide sequences of the primer sets to each type of GGTmRNAs are: type A sense 5' - CAC AGG GGA CAT ACA GTG AG-3', Antisense 5' - GAA ATA GCT GAA GCA CGC GC -3'; type B sense 5' - GGA TTC TCC CAG AGA TTG CC -3', antisense 5' - GAA GGT CAA GGG AGG TTA CC -3'; type C sense 5' - GCC CAG AAG TGA GAG CAG TT -3', antisense 5' - TCC AGA AAG CAG CTA GAG GG -3'. The condition of reaction: in advance denaturalization of 94°C for 3 min, and then PCR was performed with 30 cycles consisting of a denaturing step of 94°C 30 s, an annealing step of 58°C for 30 s and an elongation step of 72°C for 30 s. the final step at 72°C was extended to 8 min. The expected size of each PCR product is 308bp in type A, 300bp in type B, 386bp in type C. Products after gel electrophoresis by 1.3 % gelose were observed under ultraviolet radiation comparing with stander DNA.

Statistical analysis

The statistical software SPSS was used for statistical analysis, the level of significance was $P<0.05$.

RESULTS

GGTmRNA in tissues

In normal liver tissues (12 cases), type A was the main type, it was found in all livers (100 %), type B was not found, type C was found in 3 cases, the GGTmRNA expression was monogenic (type A) in 9 livers and polygenic (type A + type C) in 3 cases (Table 1).

In liver tissues with acute hepatitis, chronic hepatitis, cirrhosis or alcoholic hepatopathy, type A was the main type (81.82-93.33 %), type C was found occasionally (13.33-23.08 %), the distribution of types of GGTmRNA was similar as in normal liver tissues ($P>0.05$), type B was found in 3 cases with chronic hepatitis, 3 cases with cirrhosis, the prevalence of type B was higher than normal livers, but it was not significantly different ($P>0.05$).

In 32 cancerous tissues with HCC, type B was the main type, it was found in 28 cases, the prevalence of type B in

cancerous tissues (87.5 %) was significantly higher than in normal livers ($P<0.05$); type A was found in 15 cases, its prevalence in cancerous tissues (46.88 %) was lower than in normal livers ($P<0.05$); the type C was not significantly different between cancerous tissues and normal liver tissues ($P>0.05$). In 21 adjacent paracancerous tissues, 21 distal cancerous tissues and 11 noncancerous tissues, the main type was type A and B, the prevalence of type A (87.51 %, 90.48 %, 81.82 %) was similar as in normal livers ($P>0.05$); however the prevalence of type B (80.95 %, 76.19 %, 72.73 %) was significantly higher than in normal liver tissues ($P<0.05$); the prevalence of type C was similar as in normal livers ($P>0.05$).

In nontumor tissues of livers with hepatic benign tumor and secondary carcinoma of liver, the distribution of GGTmRNA was similar as in normal livers ($P>0.05$).

The relation between types of GGTmRNA and size of HCC: The prevalence of type A in cancerous tissues of larger sized HCC is lower than in that of smaller sized HCC, the monogenic pattern of type B tended to be found more frequently in larger sized HCC, but the difference was not significant ($P>0.05$). Table 2.

Table 1 Incidence of Different GGTmRNA types in Livers of Each Group

Group	No. of Samples	Type of GGTmRNA		
		A (%)	B(%)	C (%)
1.Normal	12	12 (100%)	0	3 (25.00%)
2.Acute hepatitis	15	14(93.33%)	1 (6.67%)	2 (13.33%)
3.Chronic hepatitis	18	16(88.89%)	3 (16.67%)	4 (22.22%)
4.Cirrhosis	11	9 (81.82%)	3 (27.27%)	2 (18.18%)
5.Alcoholic hepatopathy	13	12 (92.31%)	1 (7.69%)	3 (23.08%)
6.Hepatic benign tumor	10	10 (100%)	0	1 (10.00%)
7.Secondary carcinoma of liver	13	11 (84.62%)	0	3 (23.08%)
8.HCC	32			
Cancerous tissues	32	15 (46.88%) ^a	28 (87.5%) ^a	2 (6.25%)
Adjacent Paracancerous tissues	21	18 (85.71%)	17 (80.95%) ^a	3 (14.29%)
Distal Cancerous tissues	21	19 (90.48%)	16 (76.19%) ^a	2 (9.52%)
Noncancerous tissues	11	9 (81.82%)	8 (72.73%) ^a	1 (9.09%)

^a $P<0.05$ vs. normal liver.

Table 2 GGTmRNA and the size of HCC

	cases	Cancerous tissues type A	Cancerous tissues type B	Non cancerous tissues type B	Peripheral blood type B
Small sized HCC	8	5(62.50%)	6(75.00%)	5(62.50%)	2(25.00%)
Large sized HCC	16	7(43.75%)	15 (93.75%)	14(87.50%)	6(37.50%)
Enormous sized HCC	8	3(37.50%)	7(87.50%)	7(87.50%)	4(50.00%)

GGTmRNA in peripheral blood

In peripheral blood of 32 patients with HCC, type A was found in 2 cases (6.25 %), type B was found in 12 cases, the prevalence of type B (37.5 %) was significantly higher than normal ($P<0.05$), type C was found in 1 case (3.13 %). In peripheral blood of patients with acute hepatitis, type A was found in 2 cases. In chronic hepatitis group, type A was found in 1 case. Type B and C were not found in acute and chronic hepatitis group. In the other groups, GGTmRNA was not found. In 8 cases of small sized HCC, type B was found in 5 noncancerous tissues (62.5 %) and in peripheral blood of 2 cases (25 %). In the 8 cases, there were 3 patients with serum

AFP positive (37.5 %). Table 2.

In 21 cases of HCC with AFP positive, type B was found in peripheral blood of 8 cases (38.1 %). In 11 cases of HCC with AFP negative, type B was found in peripheral blood of 4 cases (36.36 %). Type B was not significantly different between them ($P>0.05$). Therefore the prevalence of type B in peripheral blood was not related to the prevalence of AFP.

DISCUSSION

Hepatocellular carcinoma is one of the common malignant tumor^[17-29], because of its severe malignance, quick development, early intrahepatic metastasis, mostly being combined with cirrhosis, frequent recurrence, the prognosis of HCC still remains dismal^[30-41]. By now, surgery is still the most efficient treatment for HCC, but about 70 percent of patients with HCC lost the opportunity of surgery, since they did not go to see a doctor until the tumor reached an advanced stage, and HCC reoccurred more frequently after surgery, on the other hand, HCC is not susceptible to radiotherapy, chemotherapy and other synthetic treatments^[42-44], so it is imperative to clarify the pathogenic mechanism of HCC and to find efficient methods for early diagnosis of HCC. The epidemiological studies suggested that the prevalence of HCC in patients with hepatopathy had been obviously increasing in China. At present, the pathogenic mechanisms of HCC are not well known, it is reported that the occurrence and evolvement HCC may be a process of polygenic and multiple steps, which related to polygenic expression, such as repair of DNA, signal transduction, cell cycle regulation etc^[45-52].

It is still difficult to monitor the canceration of liver cells in preneoplastic stage and early stage of HCC^[53]. If we could monitor the changes of the structure and function of some genes, we would find the patients with high risk of HCC, forecast the possibility of occurrence of HCC before cytological changes, and then we could prevent, make a diagnosis and give treatment on molecular level.

It has been reported that HCC synthesizes and secretes many proteins, polypeptides or isoenzymes such as AFP, GGT etc. they may be used as important marks for the diagnosis of HCC. GGT is closely relate to biotransformation, metabolism of nucleic acid, and the occurrence of carcinoma, it may be used as a mark for detection of bibulosity and the canceration of liver cells. In humans, the activity of GGT is at a very high level in embryo period, after birth it declines to a very low level quickly, HCC expresses a large amount of GGT and unique GGT isoenzyme. However, its mechanisms are not known. Human GGT gene is linked to a BCR gene-related segment that is located on band q11→qter of chromosome 22, maybe there is a family of GGT genes, it could suit for the various expressions of physiological aberrance and physiological state. Many studies concerning GGT complementary DNA (cDNA) sequences indicated that the cDNA sequences from fetal liver, HepG2 cells, and placenta showed identical open reading frame (ORF) consisting of 1707 nucleotide acids and GGT protein structure. The most significant difference among these cDNAs exists in the 5' -noncoding region^[54]. In the present study, polymorphisms of GGT mRNAs at the 5' -noncoding region from normal liver tissues and diseased liver tissues were analyzed with RT-PCR method.

In normal liver tissues, the main type of GGT mRNA was type A, the expression was monogenic in most cases (type A), but was polygenic in some cases (typeA+C).

In livers of acute hepatitis, chronic hepatitis, and cirrhosis, the distribution of types of GGT mRNA was nearly the same as in normal livers, but in livers of chronic hepatitis and cirrhosis, the prevalence of type B was higher than in normal livers ($P>0.05$).

In cancerous tissues with HCC, type B was predominant. In noncancerous tissues of livers with HCC, the main types were type A and B. The prevalence of type B was significantly higher in both cancerous and noncancerous tissues of livers with HCC than in normal livers and diseased livers without HCC. The prevalence of type A in cancerous tissues, but not in noncancerous tissues, was significantly lower than in normal livers and diseased livers without HCC. The prevalence of type A in noncancerous tissues of livers with HCC was similar as in livers without HCC. These results strongly suggest that the GGT mRNA expression in human liver may shift from type A to type B during the development of HCC. The prevalence of type B in noncancerous tissues of livers with HCC was significantly higher than in livers without HCC, and was similar as in cancerous tissues of livers with HCC. These suggest that the shift of the GGT mRNA may occur from the preneoplastic stage of hepatocytes. Type B was detected in livers of 3 cases with chronic active hepatitis and 3 cases with cirrhosis. It was not sure whether or not some liver cells in liver tissues of these 6 cases had developed to the preneoplastic stage or changed to cancerous cells, and whether or not these 6 patients would developed to HCC in the future. It need further follow-up study to answer these questions.

Among the patients of hepatic benign tumor and secondary carcinoma, the distribution of GGT mRNA in livers tissues but not tumor tissues was similar as that in normal livers. The results suggested that the shift from type A to type B did not exist in liver tissues with benign tumor and secondary carcinoma.

The serum level of GGT is mostly higher in patients with alcoholic hepatopathy, but the distribution of types of GGT mRNA in these liver tissues was similar as in normal liver tissues. This result suggested that alcohol did not induce the shift of GGT mRNA.

Studies about small sized HCC have been the important incident in the history of HCC in the past 20 years. Early diagnosis and treatment are the keys to increase survival rate and decrease recurrence rate. The detection of serum alpha-fetoprotein (AFP) is an important method for early diagnosis of HCC, especially in patients with high risk of HCC^[55,56]. However, the negative rate of AFP is higher in patients with small sized HCC. In present study, among the 8 patients with small sized HCC, type B was detected in noncancerous liver tissues of 5 patients, and in peripheral blood of 2 cases, however, AFP was positive in serum of only 2 patients. Moreover, in peripheral blood, type B of GGT mRNA was found in 4 of 11 HCC patients with AFP negative (36.36 %). These results suggested that the detection of unique type B of GGT mRNA may provide a useful tool for the diagnosis of the small sized HCC and HCC with AFP negative.

Since there are lots of RNA enzymes in blood plasma, RNA will be degraded by RNA enzymes as soon as it appears in plasma, so there are not dissociative GGT mRNAs in blood plasma in normal blood plasma. In this study, GGT mRNAs were not found in normal peripheral blood, however, among 32 cases of HCC, type B of GGT mRNA was found in peripheral blood of 12 cases (37.5 %). GGT mRNAs were not found in peripheral blood in other groups. So it may be inferred that cancerous cells exist in peripheral blood of this 12 patients. These results suggest that the shift of type of GGT mRNA are closely related to the development of HCC, and that analysis of GGT mRNA expression may provide a useful fool for early diagnosis of HCC.

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