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Loss of ICG uptake in the process of rat hepatocarcinogenesis correlates to the disappearance of glutathione-S-transferase alpha subunit.

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Abstract

Reduced indocyanine green (ICG) uptake is one of the functional changes of human hepatocellular carcinoma (HCC). To clarify the mechanisms of loss of ICG uptake, and determine which subunit of glutathione-S-transferase (GST), alpha or pi, plays a role in ICG transport in hepatocytes, an experimental HCC model was developed that used nodules induced by 2-acetylaminofluorene (2-AAF) administration. Many of the ICG stained nodules, which consisted of benign and borderline lesions, were GST-alpha positive. However, the percentage of GST-alpha positive cells tended to decrease according to the disappearance of ICG staining in the process of hepatocarcinogenesis. HCCs unstained by ICG were also GST-alpha negative. GST-pi, not detected in normal rat hepatocytes, appeared in an earlier stage of hepatocarcinogenesis before the disappearance of GST-alpha, and was not observed in HCCs. No significant relationship between ICG staining and GST-pi was recognized. These results suggest that GST-alpha synthesis is disturbed in the process of hepatocarcinogenesis and results in loss of ICG uptake in HCCs, and also indicate that GST-pi may be useful for early diagnosis of preneoplastic hepatocytes showing no roles in ICG transport.

KEYWORDS: hepatocarcinogenesis, indocyanine green, glutathione-S-transferase-?, glutathione-S-transferase-?

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Loss of ICG Uptake in the Process of Rat Hepatocarcinogenesis Correlates to the Disappearance of Glutathione-S-Transferase Alpha Subunit

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Reduced indocyanine green (ICG) uptake is one of the functional changes of human hepatocellular carcinoma (HCC). To clarify the mechanisms of loss of ICG uptake, and determine which subunit of glutathione-S-transferase (GST), alpha or pi, plays a role in ICG transport in hepatocytes, an experimental HCC model was developed that used nodules induced by 2-acetylaminofluorene (2-AAF) administration. Many of the ICG stained nodules, which consisted of benign and borderline lesions, were GST-alpha positive. However, the percentage of GST-alpha positive cells tended to decrease according to the disappearance of ICG staining in the process of hepatocarcinogenesis. HCCs unstained by ICG were also GST-alpha negative. GST-pi, not detected in normal rat hepatocytes, appeared in an earlier stage of hepatocarcinogenesis before the disappearance of GST-alpha, and was not observed in HCCs. No significant relationship between ICG staining and GST-pi was recognized. These results suggest that GST-alpha synthesis is disturbed in the process of hepatocarcinogenesis and results in loss of ICG uptake in HCCs, and also indicate that GST-pi may be useful for early diagnosis of preneoplastic hepatocytes showing no roles in ICG transport.

Key words : hepatocarcinogenesis, indocyanine green, glutathione-S-transferase- α , glutathione-S-transferase- π

Mechanisms of uptake of organic anions (*e.g.*, bilirubin, indocyanine green (ICG) and sulfobromophthalein (BSP) by hepatocytes have been investigated in several studies (1-6). Organic anion binding proteins (4), albumin receptors (5), or an active transport mechanism (6) are supposed to regulate the uptake. Wolkoff *et al.* (2) have identified a BSP receptor on the plasma membrane of hepatocytes. Other reports on the mechanism of ICG uptake indicate that glutathione-S-transferase (GST), a carrier protein of ICG, plays an important role in staining in hepatocytes (7-9).

ICG uptake has not been recognized in hepatocellular carcinoma (HCC) at the level of peritoneoscopic examination (10). In our first investigation we examined the relationship between ICG staining and cell atypism of rat

liver nodules induced by administration of 2-acetylaminofluorene (2-AAF) as an experimental model of human HCC. The ICG-unstained nodules were proved to be malignant by means of histological examination and DNA ploidy pattern (11).

In this report, the existence of GST-subunits (GST-alpha and -pi) of rat liver nodules obtained from the previous experiment (11) was demonstrated by immunohistochemical technique, and the relationship between ICG staining and GST-subunits was investigated in order to clarify the role of GST-subunits on ICG uptake.

Materials and Methods

An experimental model for hepatocarcinogenesis. According to the method described by Epstein *et al.* (12), 36 Wistar-strain

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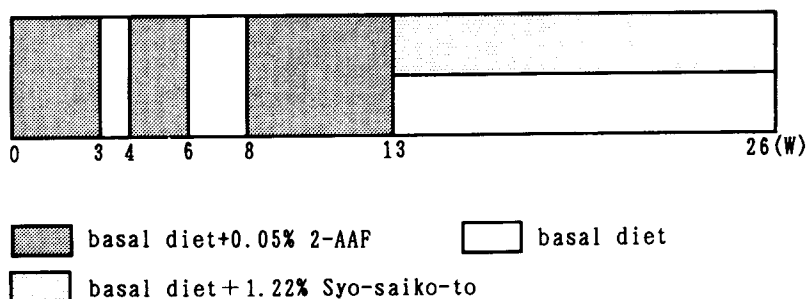


Fig. 1 Dietary regimen for 2-acetylaminofluorene (2-AAF) and Syo-saiko-to administration to induce liver nodules: Basal diet containing 0.05 % 2-AAF and basal diet alone were reciprocally fed for the period of 0-13 weeks to avoid the death from hunger. Basal diet containing 1.22 % Syo-saiko-to or basal diet alone were subsequently fed for the period of 13-26 weeks.

male rats (8-week-old, 170-180 g) were fed on diet containing 0.05 % 2-AAF for weeks 0-3, 4-6, or 8-13 orally (as initiation and promotion). Basal diet without 2-AAF were given for weeks 3-4 and 6-8. Surviving rats were divided into two groups and fed either a diet containing 1.22 % Syo-saiko-to, or basal diet for weeks 13-26 in order to generate nodules at various stages of development (Fig. 1).

ICG injection and histological diagnosis. The rats were killed as previously described (11) and according to the schedule in Table 1. They were anesthetized with ether and the portal vein was cannulated with a 24-gauge thin needle. ICG (2.5 mg/ml) was injected slowly for more than 1 min. to avoid disruptive pressure until liver surface was completely stained. The inferior vena cava was then incised at the juncture with the hepatic vein and perfused with 20 ml of saline. The largest nodules, ICG stained and unstained, were fixed in 10 % neutral buffered formalin for 7 days and embedded in paraffin. The specimens were sliced 6 μ m in thickness with a microtome. Formalin-fixed and paraffin-embedded sections were used for hematoxylin and eosin (H. E.) staining, and immunohistochemical analysis for GST-alpha and -pi.

Estimation for specificities of anti GST-alpha and -pi antibodies. According to the method of Laemmli *et al.* (13), GST from rat liver (Sigma Co. Ltd.) was separated by electrophoresis in 10 % polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) and were transferred to nitrocellulose sheets (Bio-Rad Laboratories). After blocking with 3 % bovine serum albumin-0.1 M phosphate buffered saline, pH 7.4 (PBS), blots were overlaid with anti GST-alpha antibody ($\times 100$) or anti GST-pi antibody ($\times 100$) and were incubated for 1 h at room temperature with gentle agitation. The blots were then overlaid by a 1/1000 diluted solution of peroxidase labeled swine anti-rabbit IgG for 1 h. Reactivity was detected using 3, 3'-diaminobenzidine-4HCl (DAB) (Sigma Co. Ltd.) as a substrate. Preimmune rabbit serum ($\times 100$) was used as a negative control.

Immunohistochemical staining for GST-alpha and -pi. Anti human GST-alpha antibody defined by K. Sato and anti human GST-pi (BIO PREP Co. Ltd.) rabbit polyclonal antibody were

used. Formalin-fixed and paraffin-embedded sections were deparaffinized by xylene and dehydrated by ethanol. After washing with PBS, liver sections were treated with 3 % hydrogen peroxide for 10 min and with 10 % normal goat serum for another 10 min. They were sequentially incubated with anti GST-alpha ($\times 100$) or GST-pi ($\times 1000$) antibody overnight. Avidin-biotin peroxidase complex method (ABC) was used for the staining. Positive immunoreaction was detected with 3-amino-9-ethylcarbazol (Nichirei Co. Ltd., Japan). Sections were counterstained with Mayer's hematoxylin for microscopic examination. They were also stained with H.E. Preimmune rabbit serum was used as a negative antibody control. Sections were observed by light microscope, and the immunohistochemical staining was judged to be the percentage of GST positive hepatocytes per total hepatocytes as follows; 90 % < (3+), 75 % - 90 % (2+), 50 % - 75 % (+), 25 % - 50 % (\pm) and 25 % > (-).

Statistical analysis. Significance of the differences between variables of the two groups was evaluated using χ^2 -test, and the mean values were compared with Student's *t*-test.

Results

Histological findings and ICG uptake of the nodules. Nine rats died before they could be killed. Twenty-six ICG-stained nodules (ICG(+)) and 14 ICG-unstained nodules (ICG(-)) were obtained. Histologically, 11 ICG (+) nodules were benign (42.3 %) and 15 were borderline lesions (57.7 %). ICG (-) nodules included 2 HCCs (14.3 %) and 12 borderline lesions (85.7 %) (Table 2).

Specificities of anti GST-alpha and -pi antibodies. The results of Western blot analysis for anti GST-alpha and -pi antibodies are shown in Fig. 2. Anti GST-alpha antibody strongly reacted with 27 kD protein, and anti GST-pi antibody reacted with 29 kD protein, indicating

Table 1 Time table for sacrifice of rats

Age (week)	Number of rats
7	1
9	1
10	1
12	1
13	3
17	2
18	2
21	2
22	2
24	4
26	8
Total	27

Table 2 ICG staining and histological finding in liver nodules examined

	Number of nodule lesions histologically found			
	Benign	Border	Cancer	Total
ICG (+)	11	15	0	26
ICG (-)	0	12	2	14

Table 3 Immunohistochemical staining pattern of GST- α and histological findings

GST- α staining	Number of nodule lesions histologically found		
	Benign (%)	Border (%)	Cancer (%)
-		4 (14.8)	2 (100)
\pm	2 (18.2)	5 (18.5)	
+	4 (36.3)	6 (22.2)	
2+	2 (18.2)	10 (37.1)	
3+	3 (27.3)	2 (7.4)	

(-) < 25%, (\pm) 25%~50%, (+) 50%~75%, (2+) 75%~90%, (3+) > 90%

Table 4 Relationships between GST- α staining, diameters of nodules and time from 2-AAF administration

GST- α staining	Diameter of nodules (mm)	Time from 2-AAF administration (week)
- (n=6)	9 \pm 4 (3-12)	20 \pm 5 (13-26)
\pm (n=7)	8 \pm 5 (2-15)	17 \pm 8 (7-26)
+ (n=10)	7 \pm 2 (5-11)	20 \pm 6 (12-27)
2+ (n=12)	11 \pm 5 (5-20)	21 \pm 5 (9-26)
3+ (n=5)	7 \pm 4 (2-14)	20 \pm 6 (10-26)

(-), (\pm), (+), (2+), (3+): See Table 3.

that each antibody was specific for GST subunits.

Immunohistochemical staining for GST-alpha and -pi. Early hyperplastic nodules that developed until the 10th week, 2-AAF administration were strongly positive for GST-alpha in their cytoplasm, although the surrounding tissues were negative, some showing severe fibrosis with necrosis and the destruction of the trabecular arrangement. However, the percentage of GST-alpha positive cells per total number of hepatocytes tended to decrease with the disappearance of ICG staining after 10th week of 2-AAF administration.

As shown in Table 3, the percentage of GST-alpha positive nodules (+~3+) were 81.8% (9/11) in benign tissues, 66.7% (18/27) in borderline lesions and 0% (0/2) in HCCs, respectively. The relationship between GST-alpha staining and ICG uptake was examined.

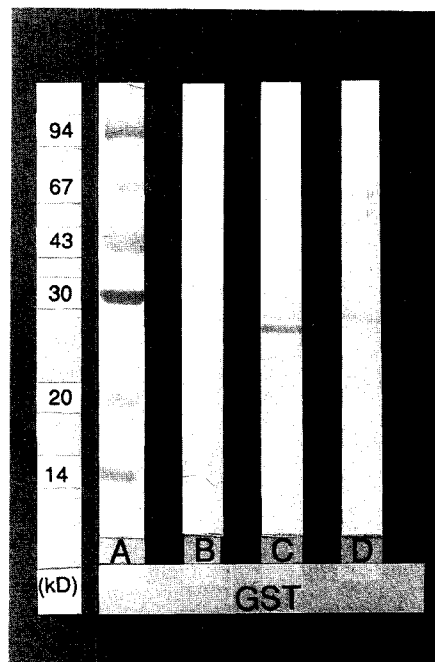


Fig. 2 Western blot analysis for anti glutathione-S-transferase (GST)-alpha and -pi: GST from rat liver (B-D) were transferred to a nitrocellulose sheet. A; Marker protein. B; The reaction to normal rabbit serum ($\times 100$) as a negative control. C; The reaction to anti GST-alpha ($\times 100$). GST-alpha specific 27kD band (arrow) was observed. D; The reaction to anti GST-pi ($\times 100$). GST-pi specific 29kD band (arrow) was observed.

GST-alpha positive nodules consisted of 81.8% (9/11) of ICG (+) benign tissues, 93.3% (14/15) of ICG (+) borderline lesions, 33.3% (4/12) of ICG (-) borderline lesions and 0% (0/2) of ICG (-) HCCs. ICG (+) nodules showed significantly larger counts of GST-alpha positive hepatocytes than ICG (-) nodules ($p < 0.01$), indicating that ICG uptake was closely related to GST-alpha staining (Fig. 3). No significant relationships between GST-alpha staining and the sizes of nodules or the time from 2-AAF administration were recognized (Table 4). The characteristic sections of nodules positive in GST-alpha are shown in Fig. 4.

In the process of the carcinogenesis, early hyperplastic nodules generated at week 7 were strongly positive of GST-pi as well as borderline lesions. However, 2 of HCC nodules did not show GST-pi positive hepatocytes. GST-pi was not expressed in normal rat hepatocytes and positive staining was only recognized in cholangioepithelial cells.

As the results shown in Table 5, the percentage of

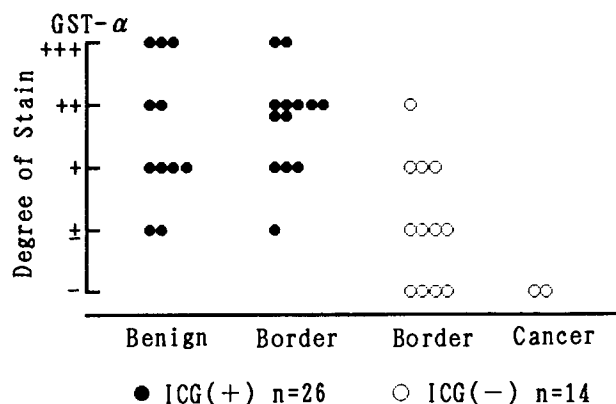


Fig. 3 The relationship between GST-alpha staining and indocyanine green (ICG) uptake of the liver nodules induced by 2-AAF: Most of stained nodules were strongly stained by GST-alpha, though most of ICG unstained nodules were not stained. It might be possible that ICG stained nodules tend to be stained with GST-alpha, too. GST: See Fig. 1.

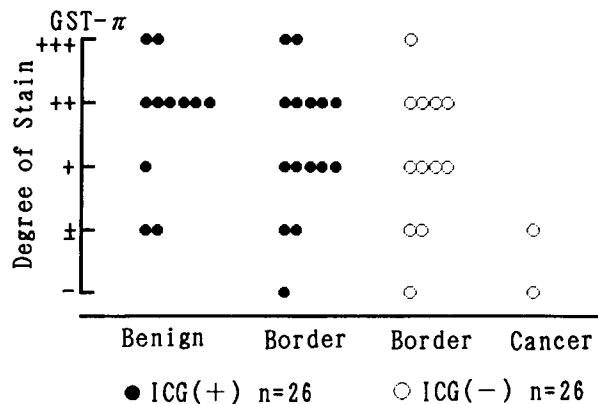


Fig. 5 The relationship between GST-pi staining and ICG uptake of the liver nodules induced by 2-AAF: Most of benign and borderline hyperplastic nodules were strongly stained by GST-pi, though all of HCCs were not stained. No significant relationships between GST-pi staining and ICG uptake were observed. GST, ICG: See Figs 1 and 3.

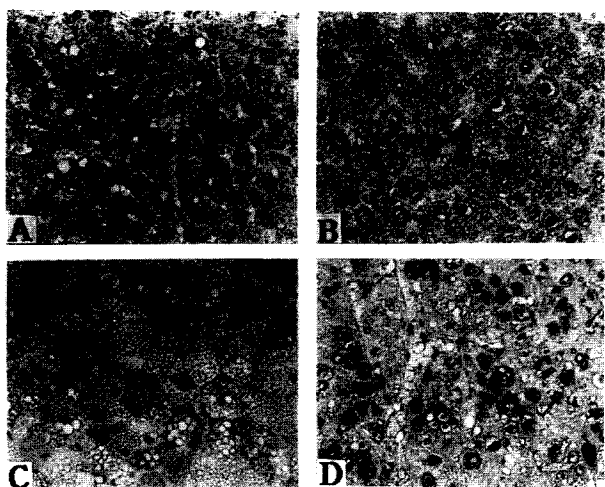


Fig. 4 Microscopic findings of liver nodules immunohistochemically stained by GST-alpha: A; Normal rat liver (7 weeks). The cytoplasm were abundant in GST-alpha. B; ICG stained benign hyperplastic nodule (10 weeks). The cytoplasm were strongly stained by GST-alpha as well as A. C; ICG unstained borderline hyperplastic nodule (26 weeks). GST-alpha was not stained in a lot of hepatocytes with fatty infiltration. D; ICG unstained hepatocellular carcinoma (HCC) (26 weeks). No hepatocytes were stained by GST-alpha. GST, ICG: See Figs 1 and 3.

GST-pi positive nodules (+~3+) were 81.8% (9/11) in benign tissues, 77.8% (21/27) in borderline lesions and 0% (0/2) of HCCs, respectively.

Table 5 Immunohistochemical staining pattern of GST- π and histological findings

GST- π staining	Number of nodule lesions histologically found		
	Benign (%)	Border (%)	Cancer (%)
-		2 (7.4)	1 (50)
\pm	2 (18.2)	4 (14.9)	1 (50)
+	1 (9.1)	9 (33.3)	
2+	6 (54.5)	9 (33.3)	
3+	2 (18.2)	3 (11.1)	

(-), (\pm), (+), (2+), (3+): See Table 3.

Table 6 Relationships between GST- π staining, diameters of nodules and time from 2-AAF administration

GST- π staining	Diameter of nodules (mm)	Time from 2-AAF administration (week)
-(n=3)	5 \pm 2 (3-8)	21 \pm 4 (17-26)
\pm (n=7)	9 \pm 7 (2-12)	18 \pm 7 (7-24)
+(n=10)	9 \pm 3 (6-18)	19 \pm 5 (13-24)
2+(n=15)	9 \pm 3 (5-12)	21 \pm 6 (12-26)
3+(n=5)	6 \pm 5 (2-14)	17 \pm 6 (10-26)

(-), (+), (\pm), (+), (2+), (3+): See Table 3.

The relationship between GST-pi staining and ICG uptake. GST-pi positive nodules consisted of 81.8% (9/11) of ICG (+) benign tissues, 80% (12/15) of ICG (+)

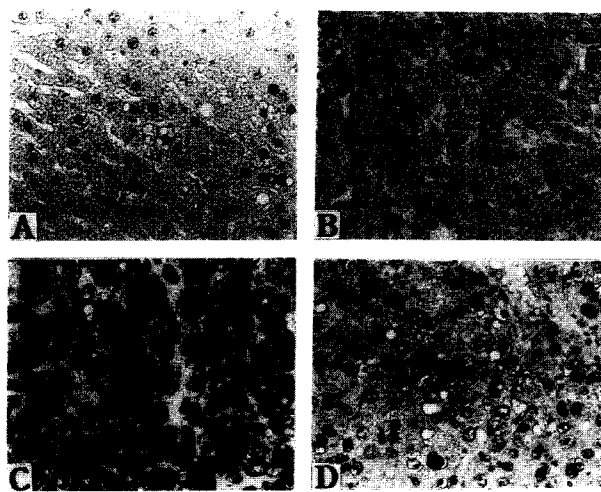


Fig. 6 Microscopic findings of liver nodules immunohistochemically stained by GST-pi: A; Normal rat liver (7 weeks). No hepatocytes were stained by GST-pi. B; ICG stained benign hyperplastic nodule (10 weeks). The cytoplasm was strongly stained by GST-pi. C; ICG unstained borderline hyperplastic nodule (22 weeks). GST-pi was stained as well as B. D; ICG unstained HCC (26 weeks). GST-pi was not stained. GST, ICG: See Figs 1 and 3.

borderline lesions, 75 % (9/12) of ICG (–) borderline lesions and 0 % (0/2) of ICG (–) HCCs (Fig. 5). Hyperplastic nodules including benign tissues without histologically atypisms and borderline lesions were strongly stained by GST-pi, suggesting no relation between their ICG uptake and GST-pi staining. In addition, significant relationships between GST-pi staining and the sizes of nodules or the time from the starting of 2-AAF administration were not recognized (Table 6). The characteristic sections of the nodules stained by GST-pi are shown in Fig. 6.

Discussion

GST is a polyfunctional enzyme constituted from subunits, and involved in detoxication for various toxins. The subunits have specific roles in conjugation with glutathione, glutathione peroxidase, and participates in leukotriene and prostaglandin metabolism (14–16). Some GST-subunits act as a ligandins which bind with organic anions (*e.g.*, bilirubin, ICG and BSP) and excrete them into bile (17). The strong expression of one of the subunits, GST-pi, in enzyme altered foci and hyperplastic nodules which are related to the preneoplastic lesions

of HCCs in humans and rats has been reported (18–19). Therefore, it is supposed to be one of the available tumor markers. In our experiment, all of benign tissues were ICG (+), all of HCCs were ICG (–) and borderline lesions consisted of both ICG (+) and ICG (–). However, the fact that ICG (–) borderline lesions showed abnormal DNA ploidy patterns (11) suggest that a loss of the ability to take up ICG is one of phenotypic changes of malignant potency.

Shimada (20) and Aikawa (21) reported that GST activity and ICG staining of the liver surface observed at the level of peritoneoscopic examination correlated well with each other, and that GST proved to be the ICG binding protein in hepatocytes. However, the identification of subunits that will bind with ICG has not been sufficiently discussed.

GST-alpha was more strongly stained in ICG (+) nodules than in ICG (–) borderline lesions or HCCs, suggesting that GST-alpha acts as a ligandin for ICG. And the decrease of GST-alpha, which might be due to the cellular transformation in the process of hepatocarcinogenesis, is an important reason for the low ICG staining of the preneoplastic nodules. On the contrary, the absence of significant correlations between GST-pi staining of ICG (+) and ICG (–) nodules excludes GST-pi as a ligandin for ICG.

It is likely that BSP is taken up by hepatocytes through BSP receptors on the liver plasma membrane (2), however, the ICG receptor has not yet been identified. In our experiment, 4 of the GST-alpha stained nodules were ICG (–). This suggests either a qualitative or quantitative dysfunction of ICG receptors maybe also play an important role in ICG staining of the nodules in the process of hepatocarcinogenesis.

Most of the hyperplastic nodules, histologically defined as benign or borderline, were GST-alpha positive in our experiment. We interpret this to mean that the decrease of GST-alpha staining is marked at the late stage of hepatocarcinogenesis. GST-pi was positive in most of the hyperplastic nodules, defined as benign or borderline, though the normal rat liver was GST-pi negative (Figs. 4 and 6). GST-alpha stained normal rat liver tissue, but GST-pi strongly stained only hyperplastic nodules, suggesting that the increase of GST-pi staining marks an earlier stage of hepatocarcinogenesis than the decrease of ICG and GST-alpha staining. Consequently, GST-pi appears to be one of the available markers that indicate preneoplastic hepatocytes.

Sato *et al.* have reported that GST-pi was abundant in preneoplastic lesions or well-differentiated HCCs in spite of its scarcity in poorly-differentiated HCCs. They also mentioned that GST-pi has already appeared at the stage of enzyme altered foci, which precedes hyperplastic nodules (18). Our experiment generated 2 types of HCC. The one weakly stained (\pm) for GST-pi was well-differentiated HCC with acinar arrangement, and the other unstained ($-$) for GST-pi was a poorly-differentiated HCC. Although further study including more subjects is necessary, our results tend to support the conclusion expressed by Sao *et al.* Further investigations into the question of whether all GST-pi positive hepatocytes become neoplastic or not are required.

In conclusion, our findings indicate that GST-alpha acts as a possible ligandin for ICG in hepatocytes and the increase of GST-pi staining appears at the earlier stage of hepatocarcinogenesis than the decrease of GST-alpha or ICG staining. These in turn suggest that GST-pi may be useful for early diagnosis of preneoplastic hepatocytes and that it does not act as ligandin for ICG.

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