Light and Electron Microscopic Immunohistochemistry of Transferrin Receptor and Its Serum Level in Human Hepatocellular Carcinoma

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ABSTRACT. The localization of transferrin receptor (TFR) in human hepatocellular carcinoma (HCC) was investigated by light and electron microscopic immunohistochemistry and its serum level was measured by an enzyme-linked immunosorbent assay. TFR expression was observed light microscopically on the plasma membrane and in the cytoplasm. Its staining pattern varied from the membranous type to the cytoplasmic type with degradation of differentiation in HCC. Electron microscopically, TFR was found in the small vesicles located below the plasma membrane, in the dilated endoplasmic reticulum, and in the Golgi complex. The vesicles containing TFR were observed below the plasma membrane in well differentiated HCC, but were found diffusely in the cytoplasm in poorly differentiated HCC. These findings correlated well with the light microscopical TFR stainings. These localizations of TFR were compatible with TFR recycling for iron uptake and TFR synthesis. They also clarified that the less differentiated an HCC was, the more activated were TFR recycling and its synthesis for increased iron demand. Therefore, this study demonstrates a correlation between TFR expression and the grade of differentiation in HCC.
The serum levels of soluble TFR in HCC cases were higher than normal, but they showed no correlation with the grade of differentiation.

Key words : transferrin receptor — human hepatocellular carcinoma — immunohistochemistry — enzyme-linked immunosorbent assay

Glycoproteins on the plasma membrane, such as asialoglycoprotein receptor and epidermal growth factor receptor,[1,2] are known to vary in their expression depending on cell proliferation or malignant change. The expression of the glycoprotein transferrin receptor (TFR) has already been investigated in several kinds of malignancies. In malignant lymphoma,[3,4] breast cancer[5] and lung cancer,[6] for example, a correlation has been found between the intensity of TFR expression and the grade of tumor differentiation. This suggests that TFR is a good indicator for the prognosis of these cancers. There have been few reports, however, on TFR expression in human hepatocellular carcinoma (HCC), and no ultrastructural studies have been done on the localization of TFR. Therefore, to clarify the details of TFR expression in HCC, its localization was investigated using light and electron microscopic

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immunohistochemistry. It has also been reported that the serum level of soluble TFR (sTFR) increases in patients with malignancy,[7] so the serum levels of sTFR were also measured in HCC cases by a sandwich enzyme-linked immunosorbent assay (ELISA), and their relationship to the grade of differentiation in HCC was examined.

MATERIALS AND METHODS

Materials

Liver tissue specimens were obtained from 4 normal adults, 6 patients with chronic hepatitis (2 with chronic persistent hepatitis (CPH) and 4 with chronic active hepatitis (CAH), 13 with cirrhosis of the liver (LC) and 19 with HCC (7 of well differentiated type, 6 of moderately differentiated type and 6 of poorly differentiated type) by fine needle biopsy with 17 or 21 gage needles, surgery or autopsy. Nine of the LC specimens were obtained from tissue surrounding HCC.

Sera for sTFR were obtained from 16 normal adults (8 males with a mean age$\pm$standard deviation (SD) of 24 ± 4, and 8 females, 23 ± 4), 14 patients with chronic hepatitis (2 with CPH and 12 with CAH), 10 with LC and 27 with HCC (11 of well differentiated type, 7 of moderately differentiated type and 9 of poorly differentiated type). The number of cases in which both tissue and sera were obtained from an identical patient was 1 with LC and 17 with HCC. All specimens were obtained under informed consent.

Methods

① ***Immunohistochemistry***

i) *Light microscopic observations*

Liver specimens were fixed with periodate-lysine paraformaldehyde (PLP) fixative,[8] washed in phosphate buffered saline (PBS : 0.01 M, pH 7.4), frozen and cut into 6 μm sections by a cryostat. The indirect immunoperoxidase method was employed as follows. After the inactivation of endogeneous peroxidase,[9] cryostat sections were incubated overnight with a mouse monoclonal antibody anti-human TFR (OKT9 : Ortho, USA, diluted 5 μg/ml in PBS containing 0.1% bovine serum albumin) as the first antibody at room temperature (temp), followed by a horseradish peroxidase conjugated goat $F(ab')_2$ anti-mouse IgG (No. 4550 : TAGO, USA, diluted 1:20 similarly) as the second antibody for 4 hr at room temp. Then the sections were incubated with diaminobenzidine (DAB) solution for 10 min, dehydrated and mounted. Observation was then performed by light microscopy. As a negative control, sections were incubated with normal mouse immunoglobulins instead of the first antibody and then were treated as described above. Alternate serial sections were stained with hematoxylin and eosin or Berlin blue stain.

ii) *Electron microscopic observations*

Serial sections for electron microscopy were made from the liver tissue used for light microscopy. These sections were treated by the indirect immunoperoxidase method described above. The sections were postfixed in 2.5% glutaraldehyde, and then were incubated sequentially with DAB solution for 30 min and with DAB solution containing hydrogen peroxide for 10 min. The stained

sections were osmicated, washed, dehydrated, embedded in Epon-Araldite and ultrathin-sectioned. Observation of the ultrathin sections was performed without additional staining under an electron microscope (JEM-100S, JEOL, Japan).

② *Measurement of sTFR*

A sandwich ELISA was performed with two kinds of mouse monoclonal antibodies anti-human TFR, Nu-TfR2 (Nichirei, Japan) and OKT9. A multi-well plate (MS-3696F, Sumitomo Bakelite, Japan) was treated with 2.5% glutaraldehyde diluted in PBS for 2 hr at room temp, washed, and then coated with 60 μl of Nu-TfR2 (diluted 2 μg/ml in PBS) overnight at 10℃. After washing, to block nonspecific binding, the plate was incubated with 100 μl of PBS containing 0.1% Na (BH_4) for 2 hr at 10℃, washed again, and incubated with 100 μl of PBS containing 1% Block Ace (UK-B25, Snow Brand Milk Products, Japan) for 2 hr at room temp. Serum samples were diluted by 40% with PBS containing 0.05% Tween and 0.4% Block Ace. Then the plate was incubated with 100 μl of the sample for 2 hr at room temp and washed. Next, the plate was incubated with 60 μl of OKT9 (diluted 2 μg/ml similarly) overnight at 10℃ and washed. Then, it was incubated with 100 μl of a horseradish peroxidase conjugated rat monoclonal antibody anti-mouse IgG_1 (No. 04-6120: Zymed, USA, diluted 1:500 similarly) for 2 hr at room temp and washed. Next, 100 μl of a coloration solution containing O-phenylene-diamine (ML-11300, Sumitomo Bakelite) was added to the wells and the plate was incubated in the dark for 10 min at room temp. Then, the reaction was stopped by adding 100 μl of 2N H_2SO_4. The optical density (OD) of the solution was measured in duplicate at 490 nm by a Microelisa autoreader (MR580, Dynatech Product, USA). The level of sTFR in serum samples was calculated from the reaction curve, which showed the relationship between the dilution of the standard serum and the standard OD. The standard serum consisted of a mixture of serum from 16 normal adults. The standard OD was measured at several dilutions of the standard serum. The normal level of sTFR was determined to be 250 units (U) based on the findings in a previous report.[10] The levels of sTFR were estimated in liver diseases and were also compared with the grade of differentiation in HCC and with laboratory data; i.e., those for serum transferrin, serum iron, total iron binding capacity (TIBC), hemoglobin (Hb), α-fetoprotein (AFP), protein induced by vitamin K absence II (PIVKA-II) and the total tumor volume calculated by abdominal ultrasonography or computed tomography. I also investigated whether iron staining of liver tissue surrounding HCC had an influence on the levels of sTFR.

③ *Data Analysis*

The correlation between TFR expression and the grade of differentiation in HCC was evaluated by the χ^2 test. The levels of sTFR in liver diseases were estimated by the Mann-Whitney test. The correlation between the levels of sTFR and the grade of differentiation and laboratory data was also assessed by the χ^2 test.

RESULTS

The grade of differentiation in HCC, the results of the immunohistochemical study, the levels of sTFR and laboratory data in 19 patients with HCC are shown in Table 1.

TABLE 1. Characteristics of patients with HCC

Case	Age and Sex	Diff	Staining				sTFR	Hb	HBV, HCV or Alc	AFP	PIVKA-II	Tumor Volume
			HCC		surHCC							
			TFR	Fe	TFR	Fe	(U)	(g/dl)		(ng/ml)	(AU/ml)	(cm^3)
1	76F	Well	M	–	ND		727	13.3	C	5	<0.06	24.5
2	70M		M	–	+	–	850	15.7	C	7	0.6	33.6
3	69F		M+C	–	+	–	619	7.4	C	71	<0.06	1.4
4	50M		M	–	–	+	562	14.7	B	30	<0.06	0.5
5	55F		M	–	ND		385	14.3	C	116	<0.06	22.5
6	81M		M	–	ND			13.0	C	265	<0.06	17.9
7	53M		M	–				12.5	C	37	<0.06	0.9
8	64M	Mode	M	–	ND		196	13.7	B+C	2,900	<0.06	4.2
9	46M		M	–			904	15.0	B	30	1.4	65.6
10	60F		M+C	–			298	13.6	C	191	<0.06	8.2
11	54M		M+C	–	+	–	491	9.6	C	150	0.2	0.4
12	77M		M+C	–	ND		791	13.8	C	12	<0.06	11.5
13	61M		M+C	–			779	12.0	C	19	1.2	65.6
14	70F	Poor	C	–	+	–	310	12.0	C	90	<0.06	46
15	69M		C	–	–	+	274	12.7	B	1,690	50	≧66
16	77M		C	–	–	+	271	13.6	C	311	10	≧66
17	55M		M+C	–	+	–	335	15.7	C	2,610	0.1	33.6
18	61M		M+C	–	+	–	1,285	11.6	C	12	27	≧66
19	71M		C	–	ND		465	14.3	Alc	10^6	8.0	≧66

Diff: differentiation
M: membranous stain
C: diffuse stain in the cytoplasm
sur HCC: tissue surrounding HCC
ND: not done
Mode: Moderate
M+C: M with slight cytoplasmic staining
Fe: Berlin blue stain
Alc: alcoholic

① *Immunohistochemistry*

i) *Light microscopic observations*

TFR expression was found to a small degree on the sinusoidal plasma membrane in normal liver (Fig. 1a) and CPH (Fig. 1b). In CAH (Fig. 1c) and LC (Fig. 1d), it became intense on the plasma membrane and was also found as small granules in the cytoplasm. There was no detectable TFR, however, on the liver tissue with positive iron staining. In well differentiated HCC, TFR was clearly observed on the plasma membrane facing the blood sinus in six of seven cases (Membranous (M) type, Fig. 2a). In the remaining case, the staining pattern was of the M + C type described later. In two of six

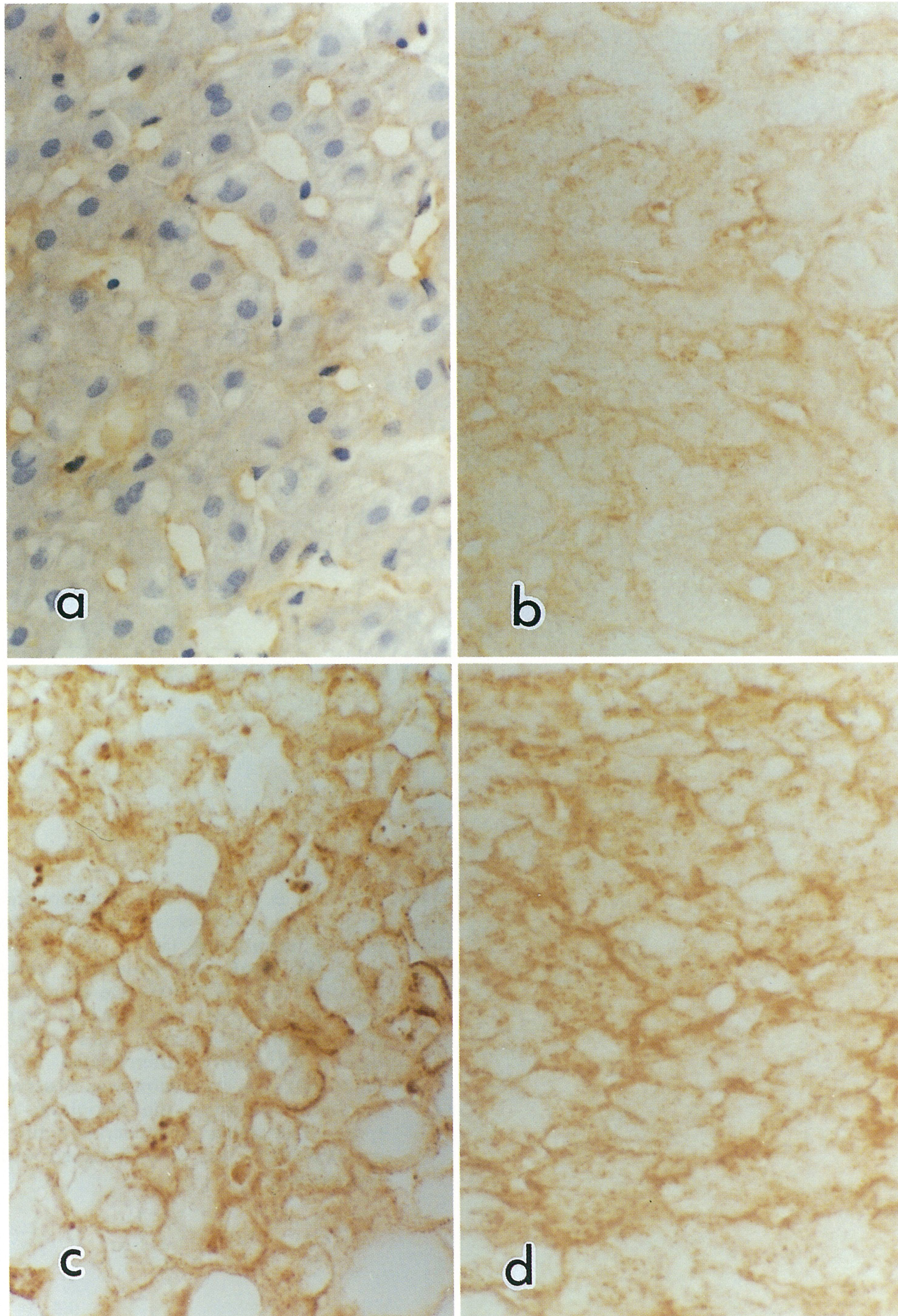


Fig. 1 TFR expression in normal liver (a, ×400) and CPH (b, ×400) was observed as weak stains on the sinusoidal plasma membrane. In CAH (c, ×400) and LC (d, ×400), however, TFR expression on the plasma membrane became more intense and that in the cytoplasm was occasionally observed as small granules. The section of normal liver was counterstained with hematoxylin.

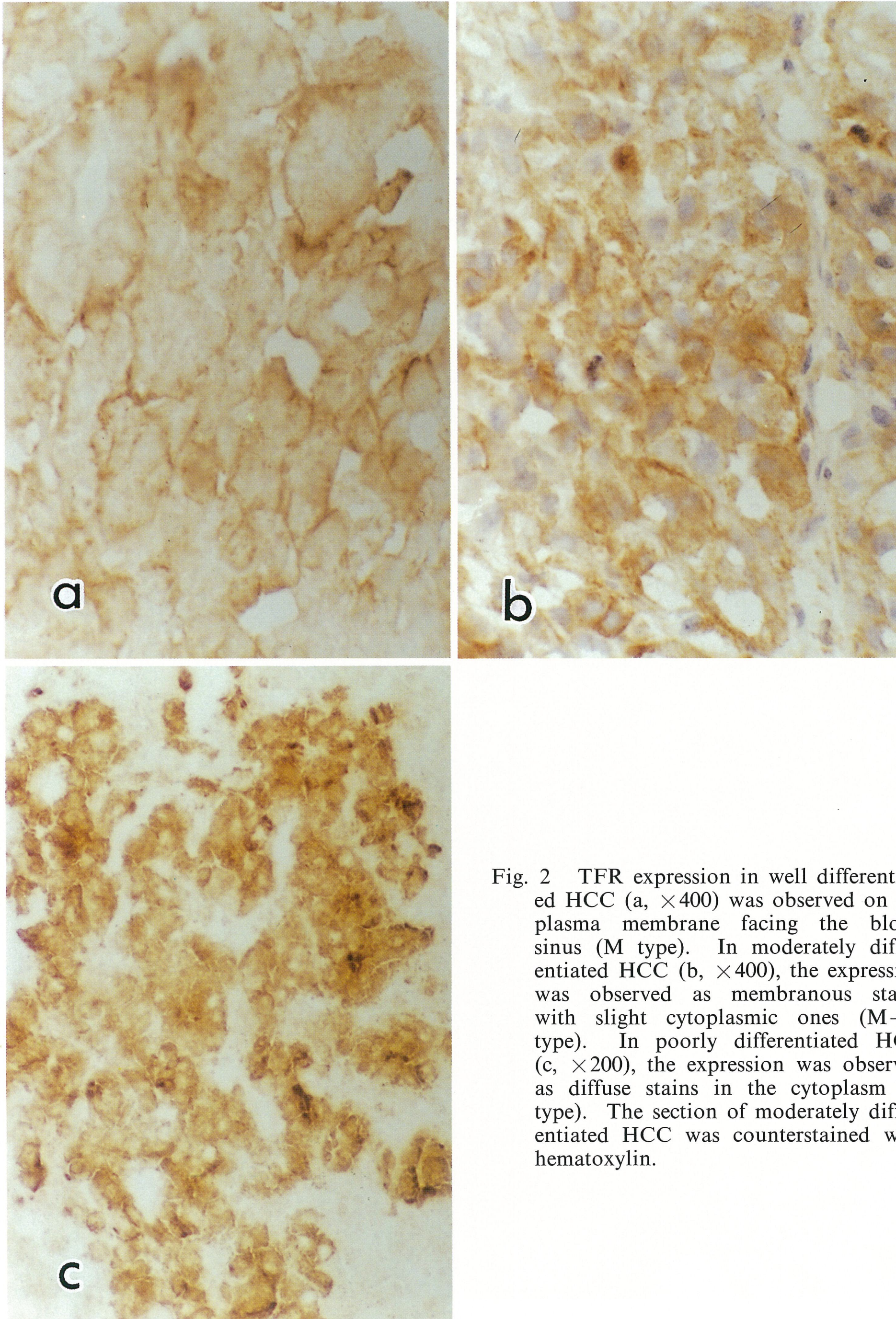


Fig. 2 TFR expression in well differentiated HCC (a, ×400) was observed on the plasma membrane facing the blood sinus (M type). In moderately differentiated HCC (b, ×400), the expression was observed as membranous stains with slight cytoplasmic ones (M+C type). In poorly differentiated HCC (c, ×200), the expression was observed as diffuse stains in the cytoplasm (C type). The section of moderately differentiated HCC was counterstained with hematoxylin.

moderately differentiated HCC, TFR was detected not only on the plasma membrane facing the blood sinus but also on the lateral plasma membrane. This pattern is also of the M type. In the remaining four cases, TFR was

observed as fine granules in the cytoplasm as well as on the plasma membrane. This staining pattern is known as the M+C type (Fig. 2b); i.e., an M type with slight cytoplasmic staining. In poorly differentiated HCC, the M+C type was observed in two of six cases. In the other four cases, TFR staining was so intense that there was diffuse staining in the cytoplasm (Cytoplasmic (C) type, Fig. 2c). Iron staining was not found on every HCC tissue. There was no detectable staining on the negative control, which confirmed the specificity of this immunohistochemistry. These findings showed that TFR expression in HCC varied from the M to the M+C to the C type depending on the degradation of differentiation in HCC. This demonstrates that there is an association between TFR expression and the grade of differentiation in HCC ($P<0.05$, Table 2).

TABLE 2. TFR expression in relation to the grade of differentiation in HCC

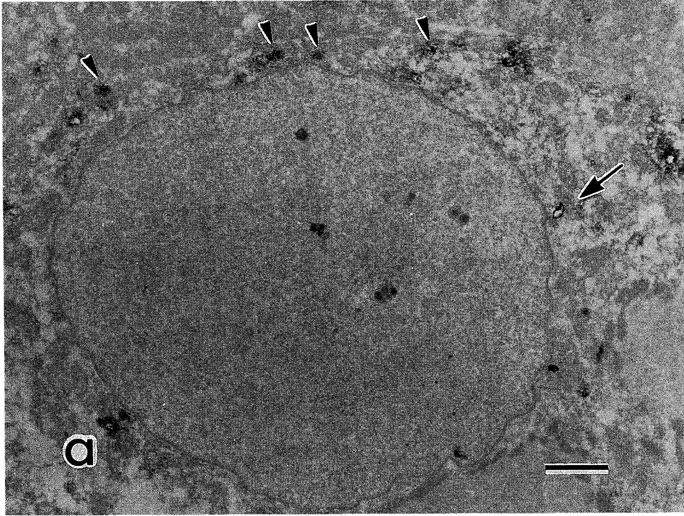
Differentiation	TFR expression			Total No. of Cases
	M	M+C	C	
Well	6	1	0	7
Moderate	2	4	0	6
Poor	0	2	4	6

A correlation between TFR expression and the grade of differentiation in HCC was indicated ($P<0.05$, χ^2 (4, 0.05) = 9.488 < χ^2 = 16.900).
M, M+C and C: TFR expression described in Table 1.

ii) *Electron microscopic observations*

Two cases of well differentiated HCC (Cases 1,3), three cases of moderately differentiated HCC (Cases 8,9,11) and two cases of poorly differentiated HCC (Cases 18,19) were observed by electron microscopy.

In the well differentiated HCC, determined to be of the M and M+C type, respectively, by light microscopy, no TFR reaction products were detected on the plasma membrane. However, they were found in the small vesicles, which were mainly located in the cytoplasm just below the plasma membrane. They were also found in the dilated endoplasmic reticulum (ER) and in the Golgi complex (Fig. 3a,b). In the moderately differentiated HCC, which were determined to be of the M, M and M+C type, respectively, by light microscopy, many small vesicles containing reaction products below the plasma membrane were observed in the M type cases (Fig. 3c). This pattern of localization was almost the same as that in the well differentiated HCC. In the case with the M+C type, however, the amount of reaction products had apparently increased in the cytoplasm (Fig. 3d). In the poorly differentiated HCC, which were determined to be of the M+C and C type, respectively, by light microscopy, both cases exhibited an increased amount of reaction products in the cytoplasm. These findings were similar to those for the moderately differentiated HCC case of the M+C type. But in the case with the C type, tumor cells often had a large number of vesicles containing reaction products. These vesicles were located not only below the plasma membrane but also diffusely in the cytoplasm (Fig. 3e). These electron microscopic findings showed that the localization of TFR also varied from the area just below the plasma membrane to the whole area of the cytoplasm and showed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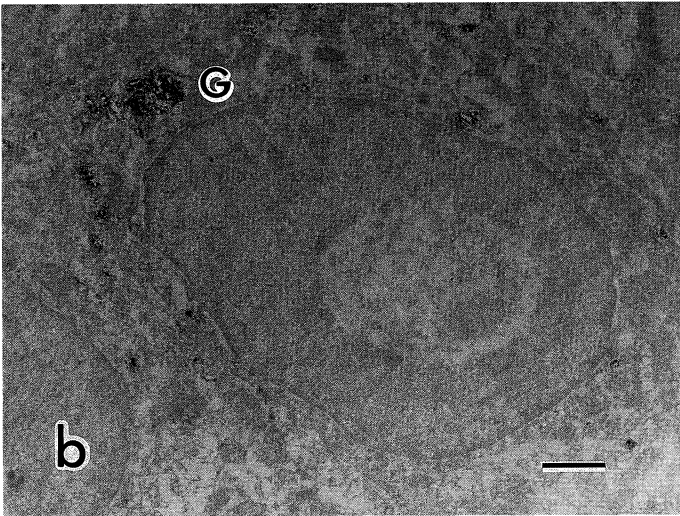
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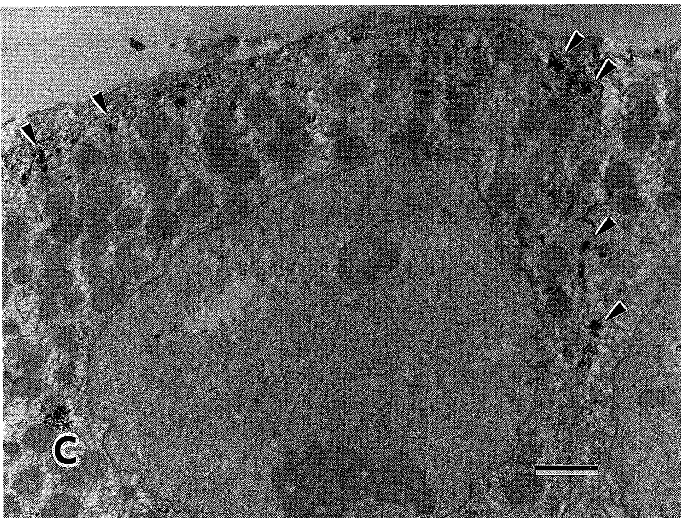
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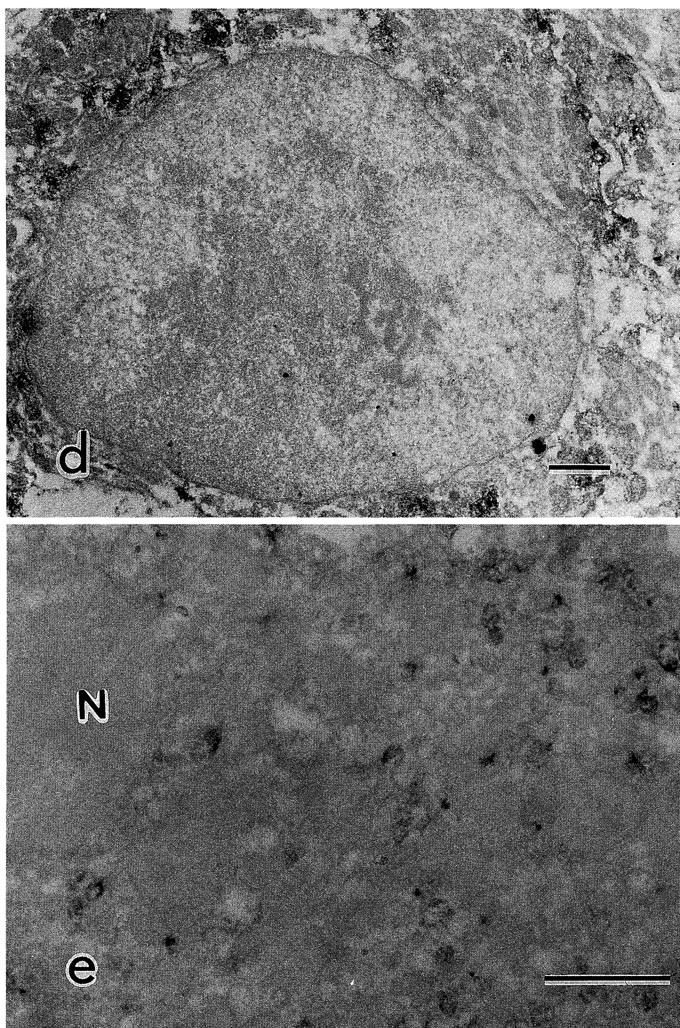

Fig. 3 In well differentiated HCC, as in Case 1 showing the M type by light microscopy, TFR reaction products were observed in the small vesicles (arrowheads, a), which were mainly located in the cytoplasm just below the plasma membrane, and TFR reaction products were also found in the dilated endplasmic reticulum (arrows, a) and in the Golgi complex ("G", b). In moderately differentiated HCC, as in Case 8 of the M type, there were a lot of small vesicles containing reaction products below the plasma membrane (arrowheads, c). This was almost the same localization as in the well differentiated HCC. In Case 11 of the M+C type, however, the amount of reaction products had apparently increased in the cytoplasm (d). In poorly differentiated HCC, as in Case 19 of the C type, tumor cells had a large number of vesicles containing reaction products, which were located not only below the plasma membrane but also diffusely in the cytoplasm (e). N: Nucleus, Bar=1 μm

some agreement with the light microscopic findings, suggesting a relationship between TFR localization and the differentiation in HCC.

② ***Measurement of sTFR***

The level of sTFR (mean±SD) was 250±75 U (male: 273±91 U, female: 227±49 U) in the serum of normal adults, 440±225 U in CH (CPH: 172±74 U, CAH: 484±247 U), 452±209 U in LC, and 622±342 U in HCC (Fig. 4). In these liver diseases, the levels of sTFR were much higher than normal ($P<0.01$) with the highest level being noted in HCC. There was no significant difference among these diseases, however. The positive rate was 57.1, 50.0 and 66.7% in CH, LC and HCC, respectively, under the condition that a level of sTFR over the mean+2SD of the normal level was considered to be positive.

With regard to differentiation in HCC, the level of sTFR was 632±206 U, 565±268 U and 656±518 U, respectively, in well, moderately and poorly differentiated HCC, and positive rate was 81.8, 71.4 and 44.4%. These findings indicate that there was no association between sTFR and differentiation in HCC despite a relationship between TFR expression and differentiation.

There was a negative correlation between the Hb concentration in HCC and the level of sTFR ($r=-0.459$, $P<0.05$, Fig. 5). There was also a negative correlation between the serum iron concentration and the level of sTFR ($P<0.1$), although statistical data were somewhat insufficient. Serum transferrin, TIBC, AFP, PIVKA-II and tumor volume showed no apparent relationship with the level of sTFR. There were no significant differences in sTFR regardless of whether iron staining of the liver tissue surrounding HCC was positive or not.

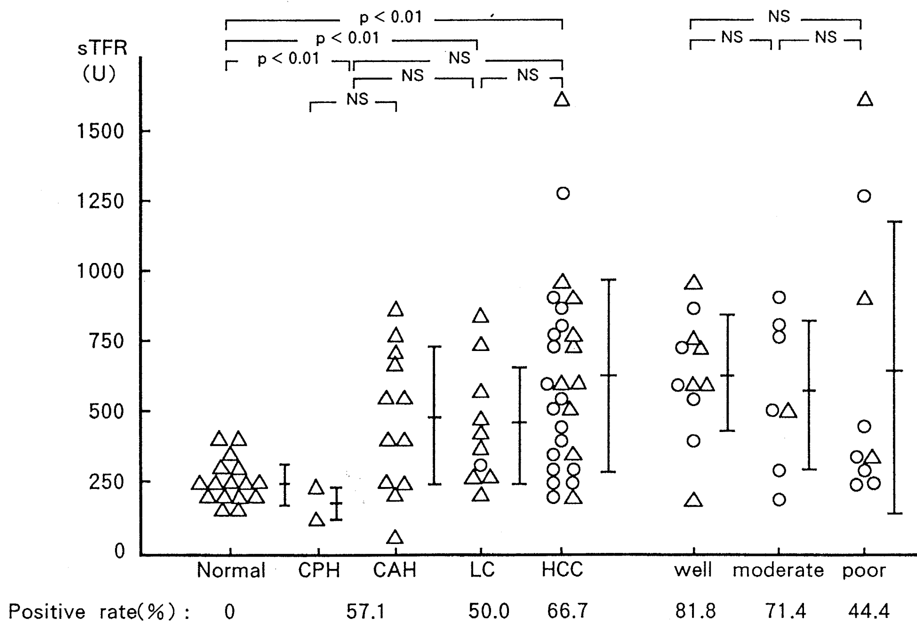

Fig. 4 sTFR in patients with chronic liver diseases
The levels of sTFR in patients with CH, LC and HCC were significantly higher than the normal ($P<0.01$). But there were no differences in the levels of sTFR among the three grades of differentiation in HCC. Immunohistochemistry was performed in the cases marked with ○ and was not performed in those marked with △.

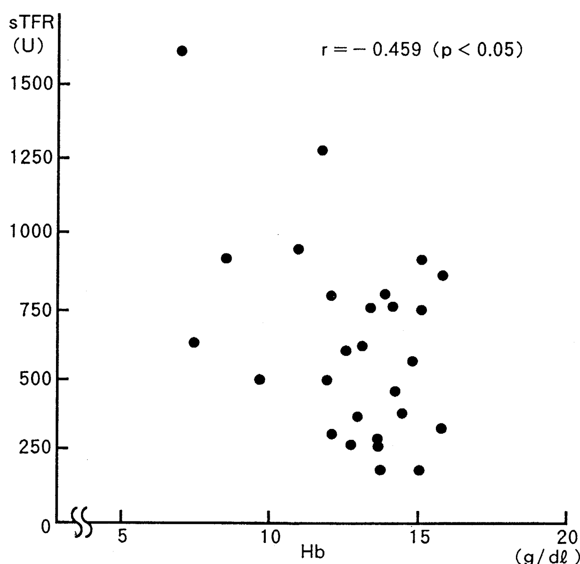

Fig. 5 Correlation between sTFR and Hb in patients with HCC
A slightly negative relationship between sTFR and Hb was present ($p<0.05$).

DISCUSSION

TFR is considered to be an iron carrier. It is endocytosed into the cytoplasm after combining with the complex of serum iron and transferrin, and then it releases iron into the cytoplasm. Then it is exocytosed to the plasma membrane, and transferrin is released into serum from TFR. In other words, TFR recycles between the plasma membrane and the cytoplasm for iron uptake. TFR is synthesized in rough ER and transported to the plasma membrane through the Golgi complex. TFR, which exists by penetrating the plasma membrane, consists of a dimer composed of two identical subunits of about 90,000 daltons.[11] The synthesis of TFR is regulated by several factors. The receptor population increases when less iron is available or cellular proliferation is elevated. It decreases when there is an oversupply of iron, or when cellular proliferation is reduced or cells are well differentiated.[12,13] Increase in TFR synthesis in HCC has been reported immunohistochemically by Sciot.[14] In that paper, the authors indicated that TFR was expressed intensely both on the plasma membrane and in the cytoplasm, but there was no correlation between TFR expression and the differentiation in HCC, although the intensity of the TFR stain varied considerably among cases. They did not provide a detailed description of what pattern of TFR staining was observed in each grade of differentiation of HCC, or of how the expression was analyzed. In addition, there has been no investigation of the ultrastructural localization of TFR in HCC. Therefore, for further examination of TFR expression, its localization in HCC was investigated by light and electron microscopic immunohistochemistry, and the serum levels of sTFR were also studied and compared with the differentiation in HCC.

In the present study, the pattern of TFR expression under light microscopic

examination was found to vary from the M to the M+C to the C type in relation to degradation of differentiation in HCC. This demonstrates that a relationship exists between TFR expression and the grade of differentiation. The present results conflict with those reported previously.[14] This might be because the categorization into M, M+C and C types was more suitable for the detection of differences in expression, since it focused not only on whether or not the expression was intense, but also on where it was intense.

Light microscopically, the M type was characterized by the pattern of TFR staining on the plasma membrane, but electron microscopically, it was found to be characterized by vesicles containing reaction products located in the cytoplasm just below the plasma membrane. The M+C type was characterized by an increase in reaction products in the cytoplasm, in addition to the findings for the M type. The C type was characterized by a large number of vesicles containing reaction products diffusely located in the cytoplasm. The fact that iron is a very important element for DNA synthesis[15,16] and that it is essential to cell proliferation could explain these findings for TFR localization. In well differentiated HCC, TFR recycles actively between the plasma membrane and the cytoplasm for increased iron demand. This recycling was thought to accord with the M type pattern or vesicles containing TFR below the plasma membrane. In moderately or poorly differentiated HCC, cell proliferation is more enhanced and there is a greater demand for iron. This causes more vivid TFR recycling, a larger amount of TFR synthesis and more active TFR transport from the site of synthesis to the plasma membrane. These movements of TFR were considered to accord with the M+C type and the C type patterns; that is, with many TFR positive vesicles and the Golgi complex in the cytoplasm.

In the next step, TFR expression in relation to iron staining was evaluated, and a negative correlation was found. As observed in some cases of CH and LC, TFR expression was positive when iron staining was negative, and the former was negative when the latter was positive. It has also been reported that TFR expression is not observed in hemochromatosis cases showing positive iron staining.[17,18] A recent genetic analysis has demonstrated that the synthesis of TFR and ferritin is controlled in a mutually opposite direction of translation on each mRNA through iron-responsive elements (IRE).[19,20] When excessive iron is present in tissue showing positive iron staining, TFR mRNA might be inhibited and TFR expression would be negative. Accordingly, the liver tissues that stained positive for iron in this study had negative TFR expression. It has also been reported that TFR mRNA in human myeloid leukemia cells increases and TFR expression becomes intense due to the growth promotion effect of G-CSF (Granulocyte-colony stimulating factor).[21] In the future, the joint study of the mRNA of TFR and ferritin and TFR expression in HCC will be important.

It has been reported that the level of sTFR in cases with HCC increases to two to four times the normal level, reflecting the total quantity of tumor cells.[10,22] This may be because the TFR existing on the plasma membrane of the tumor cells is released into serum. However, no report on the relationship between sTFR and differentiation in HCC has been published. In this study, the level of sTFR in cases with HCC significantly increased (approx. 240%) as compared with normal levels, correlating with previous reports. However, no

relationship between the level of sTFR and the differentiation in HCC was confirmed, although TFR expression in HCC tissue showed some relationship to the differentiation. These findings on sTFR may be explained by the possibility that, in HCC cases, total sTFR consists of the sTFR derived from LC tissue and that from HCC tissue. The amount of sTFR from LC tissue may be much larger than that from HCC tissue, considering that the volume of LC tissue is much greater than that of HCC tissue. In addition, the amount of sTFR from LC tissue could vary in each case with no relation to the differentiation in HCC. These characteristics of sTFR from LC tissue may hide a relationship between the level of sTFR from HCC tissue and the differentiation in HCC.

A slightly negative relationship was noted between sTFR and Hb or serum iron. In cases with HCC, these findings were understandable, since it has been reported that the level of sTFR increases in cases with iron deficiency anemia.[23)]

In conclusion, light and electron microscopic observations revealed that the pattern of TFR expression in human HCC varied from the M to the M+C to the C type in relation to degradation of differentiation. These localizations of TFR were compatible with TFR recycling for iron uptake and TFR synthesis, and they clarified that the less differentiated an HCC was, the more activated were TFR recycling and its synthesis for increased iron demand. Therefore, this study demonstrates a correlation between TFR expression and the grade of differentiation in HCC.

The serum levels of sTFR in HCC cases were higher than normal, but they showed no correlation with the grade of differentiation.

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