Hepatocyte growth factor in patients with three different stages of chronic liver disease including hepatocellular carcinoma, cirrhosis and chronic hepatitis: An immunohistochemical study

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BACKGROUND AND AIMS: The specific role of hepatocyte growth factor in liver disease is unknown. The presence and density of this factor in patients with three different stages of liver disease were investigated, with the aim of assessing its prognostic significance.

PATIENTS AND METHODS: Liver specimens from patients with chronic hepatitis (n=20), cirrhosis (n=20), hepatocellular carcinoma (n=30) and normal livers (n=20) were immunohistochemically stained to determine the presence and density of hepatocyte growth factor.

RESULTS: There were significantly more hepatocyte growth factor-positive Kupffer and Ito cells in all three diseased groups than in the control group. Also, there was significantly more positive staining in chronic hepatitis specimens than in specimens from the cirrhosis, hepatocellular carcinoma and control groups ($P<0.05$). The hepatoma cells in 10 of the hepatocellular carcinoma cases stained positive, but none of the hepatocytes in the chronic hepatitis, cirrhosis and normal liver specimens stained. It was only possible to assess nonmalignant hepatocytes adjacent to the hepatocellular carcinoma in the four resection specimens, and no staining for hepatocyte growth factor was observed in these areas. There was no statistical association between density of hepatocyte growth factor and histological activity index in chronic hepatitis, or between density of hepatocyte growth factor and grade of hepatocellular carcinoma.

CONCLUSIONS: Similar to some previous reports, this study revealed that hepatoma cells can also express this growth factor. Immunohistochemical detection of hepatocyte growth factor may prove to be a useful method of diagnosing hepatocellular carcinoma in challenging cases.

Key Words: *Chronic hepatitis; Cirrhosis; Hepatocellular carcinoma; Hepatocyte growth factor*

Pour le résumé, voir page suivante

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Facteur de croissance des hépatocytes chez des patients souffrant de maladies du foie de trois stades différents, comprenant un carcinome hépatocellulaire, une cirrhose et une hépatite chronique : une étude immunohistochimique

CONTEXTE ET BUTS : Le rôle spécifique du facteur de croissance des hépatocytes dans les maladies du foie demeure inconnu. La présence et la densité de ce facteur ont été étudiées chez des patients atteints de maladies du foie de trois stades différents, en vue d'évaluer sa signification pronostique.

PATIENTS ET MÉTHODES : Des échantillons du foie de patients souffrant d'hépatite chronique (n = 20), de cirrhose (n = 20) et de carcinome hépatocellulaire (n = 20) ainsi que de patients ayant un foie normal (n = 20) ont été colorés par immunohistochimie afin de déterminer la présence et la densité du facteur de croissance des hépatocytes.

RÉSULTATS : On a observé une quantité sensiblement plus importante dans les groupes de patients malades que dans le groupe témoin de résultats positifs pour le facteur de croissance des hépatocytes dans des cellules de Kupffer et de Ito. En outre, la coloration positive était significativement plus importante dans les échantillons d'hépatite que dans les échantillons de cirrhose, de carcinome hépatocellulaire et du groupe témoin ($p < 0,05$). La coloration des cellules de carcinome hépatocellulaire a été positive dans 10 cas sur 20, mais aucun hépatocyte dans les échantillons d'hépatite chronique, de cirrhose et de foie normal n'a été coloré. Il a été possible uniquement d'évaluer les hépatocytes non malins adjacents au carcinome hépatocellulaire dans les quatre échantillons de résection, et aucune coloration pour le facteur de croissance des hépatocytes n'a été observée dans ces régions. Aucune association statistique ne fut établie entre la densité du facteur de croissance des hépatocytes et l'indice d'activité histologique dans l'hépatite chronique ou entre cette même densité et le grade du carcinome hépatocellulaire.

CONCLUSIONS : Cette étude, comme d'autres rapports antérieurs, a révélé que les cellules d'hépatome peuvent également exprimer ce facteur de croissance. La détection par immunohistochimie du facteur de croissance des hépatocytes pourrait s'avérer une méthode utile pour le diagnostic du carcinome hépatocellulaire dans les cas difficiles.

Hepatocyte growth factor (HGF), originally described as a hepatocyte-specific mitogen, has been shown to be a potent stimulator of DNA synthesis in a variety of cell types (1-5). This growth factor also acts as a motogen, morphogen and tumour inhibitor (6). HGF-producing cells in the liver are nonparenchymal, presumably Kupffer, Ito and sinusoidal endothelial cells (7-9). This factor is also present in many organs other than the liver, and HGF messenger RNA expression is enhanced in the lung, kidney and spleen after liver damage. These findings have led to the characterization of HGF as an endocrine or paracrine factor that acts on hepatocytes and other organs (10). Levels of HGF may vary due to enhanced production or decreased hepatic clearance, or through both of these routes (10). The HGF receptor is a tyrosine kinase type encoded by the c-*met* proto-oncogene. The relationship between c-*met* and HGF expression may play an important role in hepatocellular carcinogenesis, metastasis and prognosis (11-13).

The aim of the present study was to determine the density of HGF-positive cells in three different stages of liver disease using immunohistochemical technique. Also examined were the relationship between histological activity index (HAI) and HGF staining density in chronic hepatitis (CH) specimens, and the link between HGF staining density and histological grade in hepatocellular carcinoma (HCC) specimens.

PATIENTS AND METHODS

Patients: Seventy patients with liver disease, 20 with CH (group I), 20 with cirrhosis (group II) and 30 with HCC (group III) were determined. Twenty normal liver biopsies were obtained as controls. Serological markers, abdominal ultrasonography, computed tomography and determination of alpha-fetoprotein levels were used to diagnose the three types of liver diseases, and all diagnoses were confirmed histologically. The clinical findings of patients with HCC are summarized in Table 1. The causes of the patients' liver disease are presented in Table 2. At the time of biopsy, patients with HCC and patients with cirrhosis were not undergoing any specific therapy. In the CH group, one patient had been receiving steroid treatment for eight months, and three had been receiving interferon therapy for seven months at the time of biopsy. All but four specimens (resection samples in HCC cases) were obtained by needle biopsy. Each case of HCC was graded according to the Edmondson and Steiner scheme (14). The histological activity of CH was determined according to the method of Knodell et al (15).

Histochemistry: All specimens were fixed in 10% formalin and were embedded in paraffin blocks. The blocks were then cut in serial sections of 4 to 5 µm thickness, and deparaffinized sections were prepared with hematoxylin and eosin (H&E), Masson trichrome, Manuel's reticulum stain and Perls' iron stain (16).

Immunohistochemistry: All samples were immunohistochemically analyzed using the avidin-biotin complex method. First, deparaffinized sections were incubated with primary antibodies (HGF/Scatter Factor Ab-2, Clone SBF5, monoclonal [NeoMarkers, USA]) in a 1:10 dilution at room temperature for 16 h. The antibody used was a mouse monoclonal antibody of immunoglobulin G1 isotype that was raised against a recombinant human HGF protein. It is absolutely specific for HGF, and thus does not cross-react with other members of the Skingle-serine protease group, such as HGF1/macrophage-stimulating protein, plasminogen/plasmin, tissue plasminogen activator and urinary plasminogen activator. After the incubation period, the resultant reaction was visualized using the chromogen 3,3-diamino-benzidine-tetrahydrochloride. The number of nonparenchymal cells (Kupffer, Ito and endothelial cells) per mm^2 was determined by using an ocular micrometer; the mean positive cell count for each group of specimens was then calculated (CH, cirrhosis and HCC). One slide per patient was scored, and a mean of 1,074,000 cells/mm^2 were counted in each case.

TABLE 1
Clinical findings in patients with hepatocellular carcinoma

Patient	Age (years)	Sex	Tumour diameter (mm)	Anti-HCV	HBsAg	AFP (ng/mL)	AST (U/L)	ALT (U/L)	GGT (U/L)	AP (U/L)
1	70	M	15	∅	∅	∅	108	60	369	324
2	58	M	12	∅	+	∅	∅	∅	∅	∅
3	51	M	90	∅	∅	76	45	28	270	75
4	61	M	90	∅	+	4500	28	29	63	393
5	49	M	80	∅	+	8.01	59	40	65	203
6	55	M	25	∅	+	4.06	61	87	117	170
7	64	M	100	+	∅	28.21	910	120	∅	100
8	53	M	90	∅	+	∅	34	120	∅	100
9	61	M	100	∅	+	34.860	126	50	239	176
10	44	M	35	∅	+	6.73	113	55	57	210
11*	38	M	90	∅	+	9.62	82	40	101	128
12	57	M	20	∅	+	28,000	120	90	610	310
13	76	M	30	∅	–	1.65	54	69	19	127
14*	50	F	40	∅	∅	∅	∅	∅	∅	∅
15*	48	M	25	∅	+	3828	53	66	27	101
16	70	M	30	+	∅	∅	46	21	21	131
17*	68	F	25	+	∅	28.7	89	70	100	129
18*	70	M	25	∅	+	355	23	21	28	195
19	55	F	20	+	∅	155	149	179	98	264
20	51	F	17	∅	+	513	67	30	80	161
21*	54	F	26	∅	+	800	89	49	169	121
22	56	M	100	∅	∅	∅	90	1000	∅	85
23*	53	F	50	∅	∅	1.54	42	32	92	142
24*	28	M	24	∅	+	532.5	46	128	29	159
25	51	M	30	∅	+	7.2	49	38	148	170
26	65	M	25	∅	+	∅	130	210	22	81
27	51	M	20	∅	+	47.3	75	39	46	97
28*	48	M	25	∅	+	4	40	55	48	110
29	55	F	30	∅	+	9.2	60	30	90	87
30*	60	F	40	∅	+	27	75	65	60	150

**Positive staining in malignant hepatocytes for hepatocyte growth factor; ∅ Unknown; + Positive. AFP Alpha-fetoprotein; ALT Alanine aminotransferase; AP Alkaline phosphatase; AST Aspartate aminotransferase; GGT Gamma glutamyl transferase; HBsAg Hepatitis B surface antigen; HCV Hepatitis C virus*

Statistical analysis: The Wilcoxon test and the *t* test were used to analyze the statistical relationships among the groups. P<0.05 was considered significant.

RESULTS

Immunohistochemical staining revealed diffusely scattered HGF-positive cells in all groups. While the staining was limited to nonparenchymal cells (Kupffer, Ito and sinusoidal endothelial cells) in groups I and II (Figure 1), in group III (HCC) the hepatocytes also stained (Figure 2 top, bottom). It was only possible to assess nonmalignant hepatocytes adjacent to the HCC in the four resection specimens, and these cases showed no positive staining for HGF in this area. The density of nonmalignant nonparenchymal positive cells in all groups was significantly higher than that in the control specimens. Also, the density of these cells in group I was significantly higher than that in groups II (P<0.05) and III (P<0.05) (Table 2). No association between the density of HGF-positive cells and grade of HCC (P>0.05) (Table 3), or between the density of HGF-positive cells and HAI in CH specimens (P>0.05) was found (Table 4).

TABLE 2
Causes of liver diseases in the study groups

	HCV	HBV	Alcohol abuse	Cryptogenic cirrhosis	Neonatal hepatitis	Autoimmune disease
HCC	6	22	2	–	–	–
CH	13	5	1	–	–	1
C	5	9	3	2	1	–

C Cirrhosis; CH Chronic hepatitis; HBV Hepatitis B virus; HCC Hepatocellular carcinoma; HCV Hepatitis C virus

There was cytoplasmic HGF positivity in the hepatocytes of 10 cases (33.3%) from group III. The distribution of HCC grading in these 10 patients was as follows: no grade I cases, two grade II cases, five grade III cases and three grade

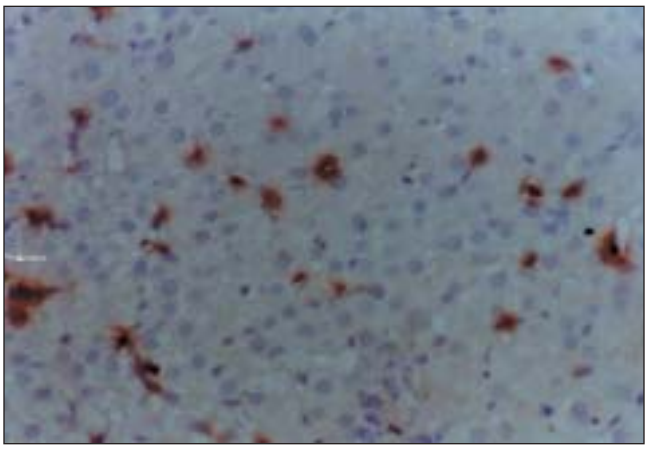

Figure 1) *Hepatocyte growth factor staining in the Kupffer and Ito cells of specimens from groups I and II (Immunoperoxidase, original magnification ×115)*

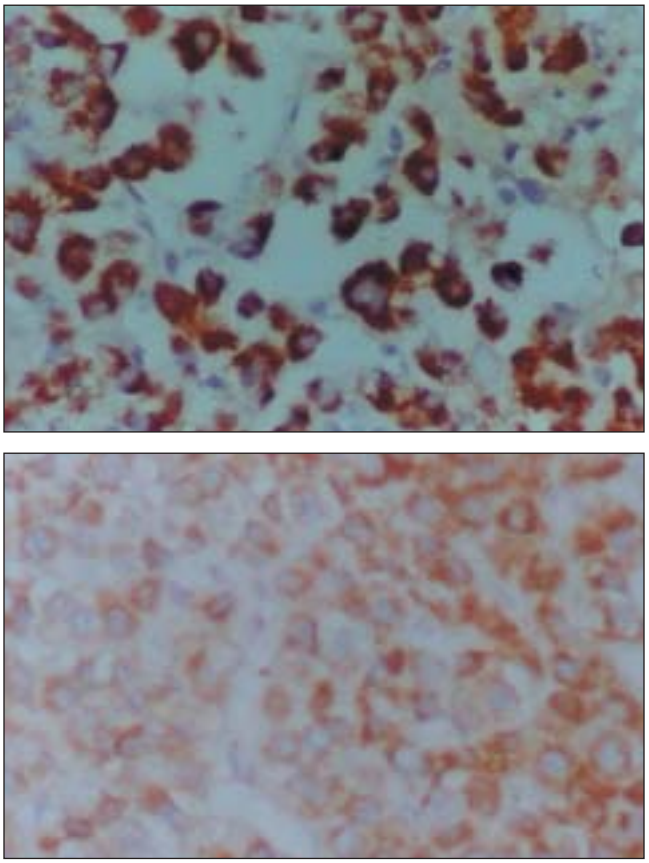

Figure 2) Top *In group III (hepatocellular carcinoma), malignant hepatocytes showed cytoplasmic staining for hepatocyte growth factor (immunoperoxidase, original magnification ×460).* **Bottom** *Cytoplasmic staining for hepatocyte growth factor in hepatoma cells (immunoperoxidase, original magnification ×230)*

IV cases (Table 5). Marked cytoplasmic staining was observed in the hepatocytes of four cirrhosis and four CH specimens, but this was identified as iron deposition (Figure 3 top, bottom). In contrast, no iron deposition was found in the 10 specimens from group III that had HGF-positive hepatoma cells (Figure 4 top, bottom). In addition, a diffuse

TABLE 3
Comparison of hepatocyte growth factor (HGF)-positive nonparenchymal cell density in groups I, II and III

Group	Number of cases	HGF-positive cells/mm² (mean ± SD)
Chronic hepatitis*	20	27±9.90
Cirrhosis	20	18.10±10.03
Hepatocellular carcinoma	30	13.03±7.90
Control	20	2.95±1.43

**Count in group I was significantly higher than the count in the other groups (t test; P<0.05)*

TABLE 4
Relationship between hepatocyte growth factor (HGF)-positive nonparenchymal cell density and grade in hepatocellular carcinoma (HCC)

Grade of HCC*	Number of cases	Density of staining for HGF antibody (HGF-positive cells/mm², mean ± SD)
I	3	16.27±7.17
II	12	9.79±6.44
III	12	14.04±8.83
IV	3	18.62±8.11

**There was no association between the density of HGF-positive nonparenchymal cells and grade of HCC (Wilcoxon test, P>0.05)*

TABLE 5
Relationship between histological activity index (HAI) and hepatocyte growth factor (HGF)-positive nonparenchymal cell density in patients with chronic hepatitis

HAI*	Number of cases	Density of staining for HGF antibody (HGF-positive cells/mm², mean ± SD)
Very low	10	21.91±7.42
Low	8	33.07±11.61
Medium	–	–
High	2	25.10±7.21

**There was no association between the density of HGF-positive nonparenchymal cells and HAI (Wilcoxon test; P>0.05)*

pattern of oval-shaped noncytoplasmic globules that stained positive for HGF was noted in two of the cirrhosis specimens. These two cases exhibited severe cholestasis on H&E staining (Figure 5 top, bottom). Two patients in the cirrhosis group showed cholestasis as well, but the degree of cholestasis was minimal, and there was no staining for HGF in these two patients.

DISCUSSION

HGF is known to be a multipotent growth factor. Previous work has shown that the expression of HGF is associated with tissue regeneration, wound healing, normal tissue growth, tumour progression, embryogenesis and tumour invasion (13,17,18). The level of expression is increased in some patients with chronic active hepatitis (19).

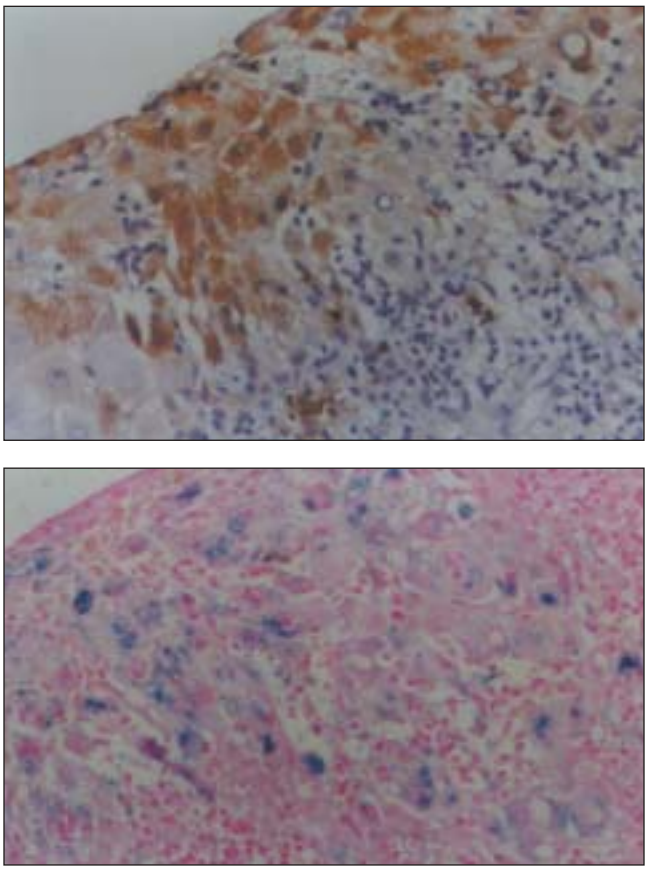

Figure 3) Top *Marked cytoplasmic staining for hepatocyte growth factor in the nonmalignant hepatocytes of a chronic hepatitis specimen (immunoperoxidase, original magnification ×115).* **Bottom** *The staining reflects iron deposition (Perls' iron, original magnification ×115)*

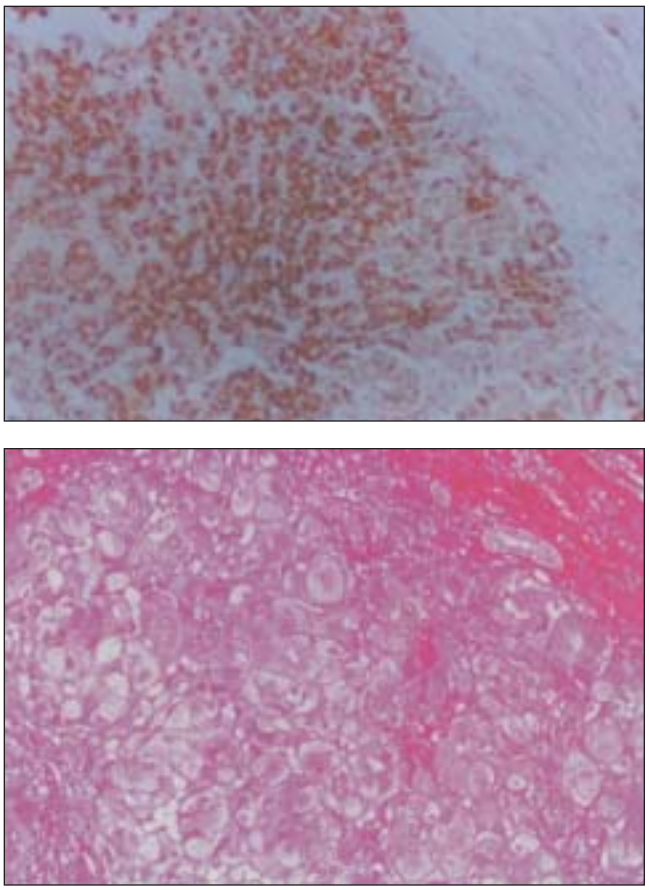

Figure 4) Top *Cytoplasmic staining for hepatocyte growth factor in malignant hepatocytes in a case of hepatocellular carcinoma (immunoperoxidase, original magnification ×46).* **Bottom** *No iron deposition is seen (Perls' iron, original magnification ×230)*

Investigation has proved that serum HGF levels are linked to HAI in this group (10,19). Our findings concurred with some of these earlier results in that immunohistochemical staining for HGF was of significantly higher density in CH specimens than in those from cirrhosis and HCC patients ($P<0.05$ and $P<0.05$, respectively); however, we found no association between density of HGF-positive cells and HAI ($P>0.05$). This could be a reflection of the small number of CH patients studied. These findings are in line with previous results indicating that hepatocyte growth factor is produced and expressed by nonparenchymal cells in nonneoplastic liver disease.

Peptide growth factors are thought to be involved in the pathogenesis of hepatic fibrosis, and overexpression of transforming growth factor beta-1 (TGF-β1) has been proposed as an important initial step. Although HGF is expressed in CH, elevated TGF-β1 levels suppress HGF expression and induce hepatic fibrosis (20). All but one of the CH cases exhibited minimal fibrosis; thus, we contend that TGF-β1 levels were low and did not suppress HGF in these patients.

A particularly interesting aspect of this study was the detection of HGF in HCC cells. As noted earlier, HGF is known to be expressed in nonparenchymal cells, such as Kupffer, Ito and endothelial cells, both in normal tissue and in various liver diseases (11,19,21-23). Most articles on this subject have stated that HGF may be detected in nonparenchymal cells within HCC nodules, but that neoplastic hepatocytes do not produce this factor (11,19,21,24-26). In contrast, other reports have shown that some hepatoma cells do stain with HGF (13,27). One of these was an experimental study in rats, but the other was an investigation of human liver tissue. The latter authors reported that five of their six HCC cases had hepatoma cells that stained HGF-positive, and concluded that these cells were expressing HGF (13). Similarly, previous reports have documented HGF production by some carcinoma cells (lung carcinoma and glioblastoma cells). In our investigation, hepatoma cells in 10 of the 30 HCC cases stained positive for HGF. To confirm the specificity of our result, we ruled out the presence of pigments known to cross-react with HGF, including iron, lipofucsin and bile.

Concerning other possible confounding findings, careful analysis of the four cirrhosis and four CH specimens that showed marked cytoplasmic staining for HGF in the hepatocytes revealed that this staining was due to iron deposi-

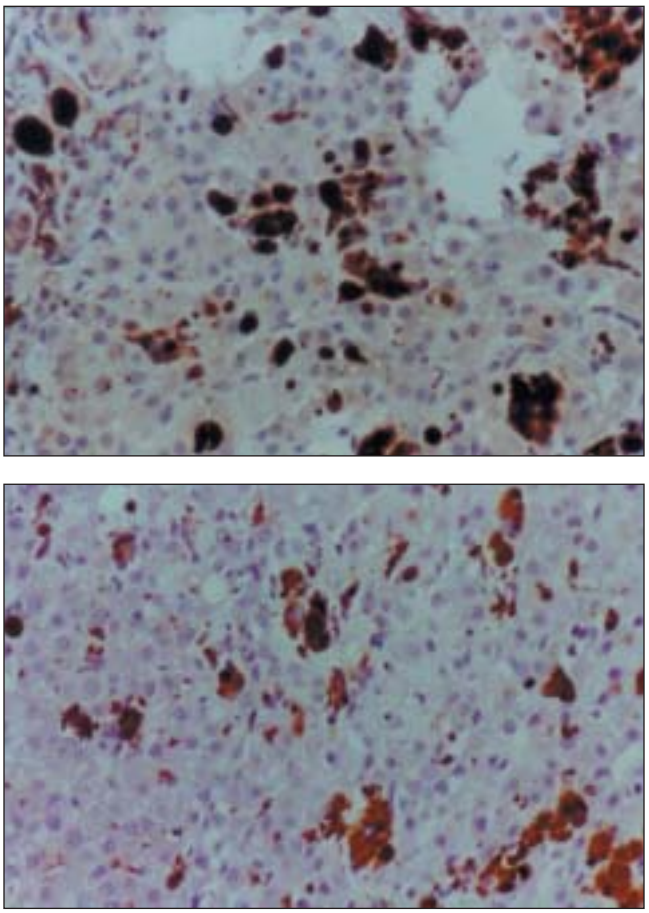

Figure 5) Top *A diffuse pattern of oval-shaped noncytoplasmic globules showed marked staining for hepatocyte growth factor (immunoperoxidase, original magnification ×115).* **Bottom** *Severe cholestasis was exhibited (hematoxylin and eosin stain, original magnification ×115)*

tion. In addition, we noted a diffuse pattern of oval-shaped noncytoplasmic globules that stained positive for HGF in two of the cirrhosis specimens. These two cases exhibited severe cholestasis on H&E staining, whereas this was not observed in the other slides. We concluded that, before hepatocyte staining with HGF can be confirmed as a valid finding, hemosiderosis and cholestasis must be ruled out.

TABLE 6
Density of hepatocyte growth factor (HGF)-positive cells in hepatoma cells according to grade of disease

Grade of hepatocellular carcinoma	HGF-positive hepatocytes/mm^2
I (n=0)	–
II (n=2)	20, 7.6
III (n=5)	10, 11.4, 13.7, 21.8, 5.75
IV (n=3)	23.3, 23.3, 9.25

On this basis, we examined the immunohistochemically stained and H&E-stained specimens simultaneously. Regarding a possible link between HGF positivity and treatment received at the time of biopsy, none of the patients in the HCC and cirrhosis groups were undergoing specific therapy, and only three patients in the CH group were receiving interferon. On this basis, we believe that the HGF expression that we observed was likely not influenced by any therapy.

Concerning potential relationships between HCC grade and HGF positivity, we observed that none of the 10 HGF-positive HCC cases exhibited grade I differentiation. However, our analysis revealed no association between density of HGF-positive cells and grade of HCC (Table 6). We think that there may be a connection between the reasons why hepatoma cells in grade I disease do not stain for HGF and why normal and non-neoplastic liver tissues do not stain.

In summary, our results should be considered preliminary. While they concur with the findings of some previous reports, other studies have failed to detect HGF in malignant cells. Our results may be related to the antibody that we applied. To our knowledge, our laboratory is the first to have used this antibody. If further research proves that hepatoma cells do produce this growth factor, immunohistochemical staining for HGF may be a valuable method for diagnosing well differentiated HCC. More investigation is required to determine the possible mechanisms of HGF staining in HCC.

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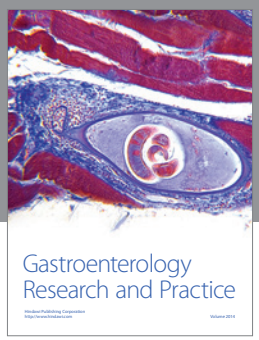

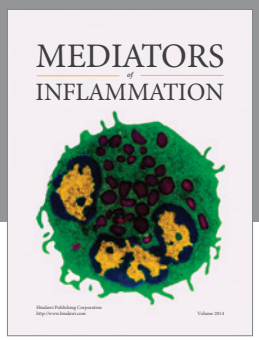

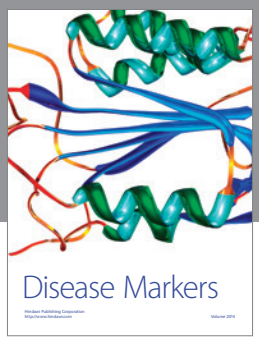

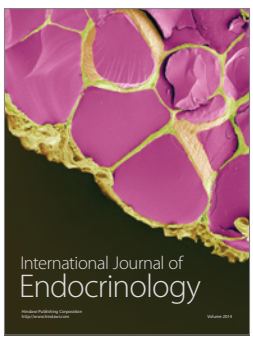

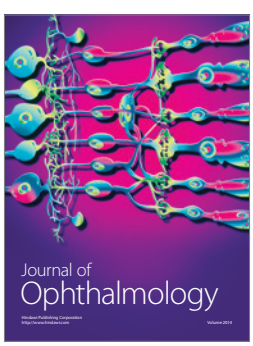

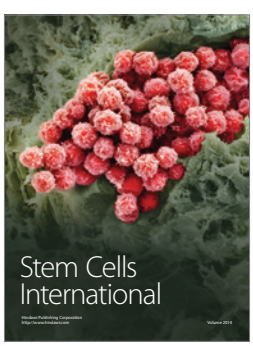

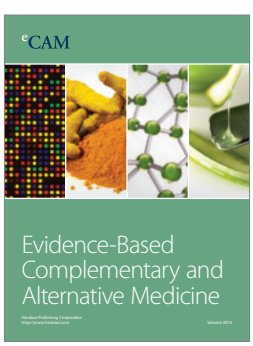

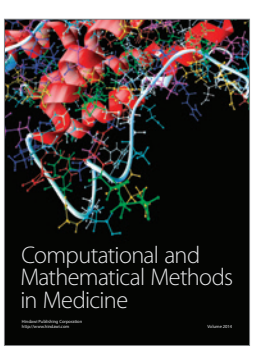

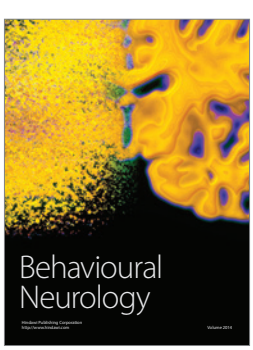

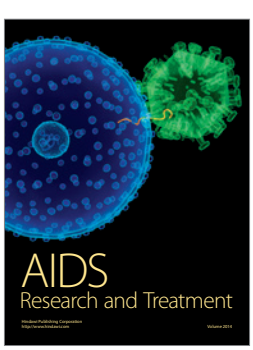

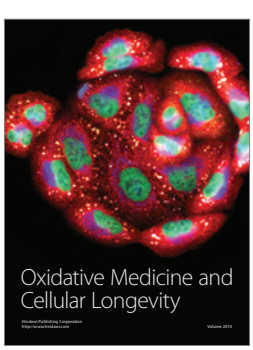

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