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LIVER CANCER

Nerve growth factor involvement in liver cirrhosis and hepatocellular carcinoma

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Abstract

AIM: To define NGF (nerve growth factor) and its high-affinity receptor trkA^{NGF} presence and distribution in fibrotic liver and in HCC, and to verify if NGF might have a role in fibrosis and HCC.

METHODS: Intracellular distribution of NGF and trkA^{NGF} were assessed by immunohistochemistry and immuno-electron microscopy in liver specimens from HCC, cirrhosis or both. ELISA was used to measure circulating NGF levels.

RESULTS: NGF and trkA^{NGF} were highly expressed in HCC tissue, mainly localized in hepatocytes, endothelial and some Kupffer cells. In the cirrhotic part of the liver they were also markedly expressed in bile ducts epithelial and spindle-shaped cells. Surprisingly, in cirrhotic tissue from patients without HCC, both NGF and trkA^{NGF} were negative. NGF serum levels in cirrhotic and/or HCC patient were up to 25-fold higher than in controls.

CONCLUSION: NGF was only detected in liver tissue with HCC present. Intracellular distribution suggests paracrine and autocrine mechanisms of action. Better definition of mechanisms may allow for therapeutic and diagnostic/prognostic use of NGF.

Key words: Nerve growth factor; Hepatocellular carcinoma; Liver cirrhosis; Confocal microscopy; Transmission electron microscopy

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INTRODUCTION

NGF (nerve growth factor) is a prototypical member of the neurotrophin family essential for survival, differentiation, and maintenance of neuronal cells in the central and peripheral nervous system[1]. In recent years, many findings have indicated that NGF could also have a role outside the central and peripheral nervous system. In particular, it may be involved in lung and skin tissue repair[2] as well as in allergic inflammation and fibrosis[3]. Increased levels of circulating NGF were observed in several autoimmune, chronic inflammatory and fibrotic disorders[4,5]. Numerous data also indicate that NGF is involved in tumour growth, invasion and metastasis[6-14].

Two types of receptors mediate NGF effects: trkA^{NGF}, the high-affinity receptor with protein kinase activity, specific for NGF, and p75^{NTR}, the low-affinity glycoprotein receptor, also binding other neurotrophins[15,16]. Most of the biological activities elicited by NGF are mediated by binding to trkA^{NGF} receptor[17,18]. TrkA^{NGF}, p75^{NTR} receptors or both, together with NGF, are expressed in various cancers, including lung[7], breast[12] and prostate cancer[10,11,13], suggesting that the NGF autocrine or paracrine pathway may have a role in tumorigenesis. NGF is also shown to be over expressed in lung and skin fibrotic process[2] as well as in liver during experimental fibrotic injury[19]. However, the role of NGF in tissue remodelling, fibrotic process and cancer progression is still controversial.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, showing a rapid progressive clinical course, poor response to pharmacological

treatment and a severe prognosis[20,21]. A major risk factor for HCC is hepatitis C virus (HCV)-related cirrhosis[22-25]; many chronically infected patients remain asymptomatic for a long period, with liver cirrhosis developing after approximately 30 years[26,27]. The lack of predictive markers that can detect the beginning of liver cirrhosis in chronic HCV patients may also contribute to the late diagnosis of HCC, its progression and the poor prognosis.

The expression of NGF and its receptors in liver tissue during fibrotic injury and HCC has been previously investigated mostly in animal models with contradictory results. Some authors demonstrated that hepatocytes expressed NGF but not trkA^{NGF} during profibrotic liver injury, or in early preneoplastic lesions and HCCs. TrkA^{NGF} was only expressed in the walls of arteries associated with tumors[28,29]. These data suggest that in hepatocytes NGF may not be an autocrine factor, but may rather act in a paracrine fashion. On the contrary, other authors[19] reported the presence of trkA^{NGF} mRNA, but not of NGF mRNA in hepatocytes of fibrotic rat liver. It is not clear from the literature which cell types (apart the hepatic stellate cells) constituting the whole liver tissue are actually involved in the cross-talk mediated by NGF during liver tissue remodelling processes and HCC progression.

In this study we investigated the expression of NGF and its high-affinity receptor trkA^{NGF} in patients suffering from HCC, cirrhosis or both. This was performed to verify if the expression of this neurotrophin may be correlated with tissue remodelling processes and HCC progression. We also studied the expression of NGF and trkA^{NGF} in the different cell types inside the fibrotic and HCC tissues, analyzing the intracellular distribution in each cell type at an ultrastructural level, attempting to clarify their involvement in the hepatic damage. Circulating NGF levels were also measured.

MATERIALS AND METHODS

Human liver specimens

20 human liver specimens, taken for diagnostic purposes or resected before transplantation, were used in this study: 5 near normal liver biopsy specimens, 4 with cirrhosis without HCC from transplanted patients (Child-Pugh A), 11 with cirrhosis and HCC post-hepatitis C [Child-Pugh A (n = 4); Child-Pugh C (n = 7)]. All the patients had voluntarily signed a written consent to participate the study. The diagnosis was based on histopathologic examination of routinely processed tissue together with clinical and laboratory data. Each specimen was received fresh, and fixed in part in 10% buffered formalin solution for immunohistochemistry, and the other part in 1% or 2.5% glutharaldehyde for immunogold labelling or morphological analysis by transmission electron microscopy observation, as described below.

Criteria used for identification of immuno-positive cell types in liver tissues

Cell types in liver tissues from cirrhotic and HCC patients have been identified on the basis of microscopic and ultramicroscopic morphological feature. In particular: (1) Endothelial cells: elongated narrow cells composing the wall of sinusoids; (2) Kupffer cells: macrophage cells, exhibiting considerable phagocytic ability, bulging into sinusoidal lumen; (3) Biliary epithelial cells: tightly arranged cuboid or columnar cells, constituting the wall of bile ducts, lying on a basement membrane and bearing microvilli on the luminal surface; (4) Spindle-shaped cells: elongated fibroblast-like cells embedded in fibrous tissue, possessing characteristics of myofibroblast, as shown by positive staining for smooth muscle actin (SMA).

Immunohistochemistry and Confocal Laser Scanning Microscopy (CLSM)

Expression of NGF and trkA^{NGF} in normal liver, cirrhotic and HCC tissues, obtained as described above, was investigated by Confocal Laser Scanning Microscopy (CLSM) using an indirect immunofluorescence technique. Immuno labelling was performed on sections obtained from paraffin embedded tissues using rabbit polyclonal antibodies against NGF (Chemicon Int., Temecula, CA, USA) or trkA^{NGF} (Santa Cruz Biothech., Santa Cruz, USA). Primary antibodies detection was obtained by a reaction with TRITC-conjugated goat anti-rabbit IgG. To test the specificity of the immunoreaction, for each liver specimen examined and on a section close to that used for NGF or trkA^{NGF} staining, negative controls were performed by substitution of primary antisera with non-immune rabbit serum. Fluorescently labelled samples were imaged by the confocal microscope LEICA TCS 4D (Leica, Heidelberg, Germany) supplemented with an Argon/Krypton laser and equipped with 40 × 1.00-0.5 and 100 × 1.3-0.6 oil immersion lenses. The excitation/emission wavelengths employed were 488 nm/510 nm, for green auto-fluorescence (used to visualize liver tissue morphology), and 568 nm/590 nm for TRITC-labelling. During the observation, both fluorescent signals were obtained simultaneously. To better visualize the intracellular distribution of NGF or trkA^{NGF} along the thickness of tissue sections, confocal sections were taken at intervals of 0.5 μm, a 3D reconstruction image for each fluorescent signal was recorded, and merged images of the two signals were obtained using the confocal microscope software.

To determine the nature of spindle-shaped cells, double staining using rabbit polyclonal antibodies against NGF (Chemicon Int.) and mouse monoclonal antibody against smooth muscle actin (SMA, Sigma-Aldrich Co., St. Louis, Mo, USA) was performed. Anti-SMA detection was obtained by a reaction with Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Molecular Probes Inc., Eugene, OR).

For the assessment of staining intensity, a minimum of three section for each sample were examined and the different level of positive immunoreaction was evaluated on the basis of number of positive cells and fluorescence intensity. The following criteria were used for evaluation of staining: (-), same as the background obtained in control performed by substitution of specific anti-NGF or trkA^{NGF} antibodies with non-immune rabbit serum; (±), moderately higher than the background; (+), higher than the background; (++), much higher than the background.

Table 1 Expression of NGF and trkA^{NGF} HCC and cirrhotic tissues and in normal liver

N°11 Patients			Cirrhotic liver		HCC	
ID#	Etiology	Child-Pugh	NGF	trkA^{NGF}	NGF	trkA^{NGF}
2	HCV	A	++	++	+	+
4	HCV	A	+	+	±	±
5	HCV	A	+	+	+	±
10	HBV	A	±	+	±	±
3	HCV	C	++	++	+	+
11	HCV	C	±	+	+	+
14	HCV/HBV/HDV	C	+	++	±	+
16	HCV	C	+	±	+	+
15	HCV	C	na	na	+	++
17	HCV	C	na	na	±	++
18	HCV	C	na	na	±	+

N° 4 Patients			Cirrhotic liver	
ID#	Etiology	Child-Pugh	NGF	trkA^{NGF}
20	HCV	C (transplantation)	-	-
21	HCV	C (transplantation)	-	-
22	HCV	C (transplantation)	-	-
23	HCV	C (transplantation)	±	-

N° 5 patients	Normal liver	
ID#	NGF	trkA^{NGF}
1	-	-
7	-	-
8	-	-
9	-	-
13	-	-

na: not available (HCC tissue without cirrhosis).

For histological observation, sections obtained from paraffin embedded tissues and close to that used for immunohistochemistry were stained with haematoxylin-eosin (HE) and observed by an optical microscope.

Transmission Electron Microscopy (TEM)

For ultrastructural observation, samples of normal liver, cirrhotic and HCC tissues were fixed with 2.5% glutharaldehyde in 0.1 mol/L Millonig's phosphate buffer (MPB), and then post-fixed with 1% OsO_4 in the same buffer. Samples were dehydrated in increasing concentrations of ethanol and embedded in Spurr epoxy resin (Agar Scientific LTD, Stansted, Essex, UK). For immuno-electron microscopy, tissue samples were fixed with 1% glutharaldehyde in 0.1 mol/L MPB, dehydrated in increasing ethanol concentrations and embedded in LR White acrylic resin (Agar Scientific LTD). Immunogold labelling was performed on ultrathin sections using the same rabbit polyclonal antibodies against NGF or trkA^{NGF} used for immunohistochemistry. Negative controls were performed by substitution of primary antisera with non-immune rabbit serum. Detection of primary antibodies was obtained by the reaction with 10 nm gold particles-conjugated goat anti-rabbit IgG. For morphological and immunogold labelling observation, ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Philips CM12 transmission electron microscope operating at 80 kV.

NGF determination

Circulating NGF levels were measured in sera from 27 patients with a documented cirrhosis, HCC or both, by a modified highly sensitive two-site immunoenzymatic assay (ELISA), using anti-NGF antibodies (clone 27/21, Chemicon Int.)[30,31]. This assay specifically identifies human NGF but not brain-derived neurotrophic factor, with a detection limit of 0.5 pg/mL.

RESULTS

Expression of NGF in liver biopsies

The results of NGF and trkA^{NGF} immunostaining in all the human liver specimens examined are summarized in Table 1.

Analysis of NGF distribution by immunohistochemistry and CLSM observation indicated that all 11 patients with cirrhosis and HCC were positive, while the 5 normal liver biopsy specimens as well as the 4 samples from transplanted patients with cirrhosis without HCC, were negative. In HCC tissues NGF was detectable in a high number of cells at different levels of intensities, depending on the patient (Table 1, Figure 2) but never in normal liver tissue (Table 1, Figure 1). Interestingly, in liver from patients with cirrhosis (Child-Pugh C, undergoing transplantation) but without HCC, NGF and trkA^{NGF} were negative (Table 1 and Figure 3A$_2$ and 3A$_3$). Conversely NGF and trkA^{NGF} were markedly positive in patients with cirrhosis that had evolved into HCC, already at early staging (Child- Pugh A; Table 1 and Figure 3B$_2$ and 3B$_3$).

Furthermore, both the number of positive cells and the intensity of immunoreaction were significantly elevated in cirrhotic tissues with respect to the HCC tissues obtained from the same liver (Table 1 and Figure 4). Moreover in HCC tissues, NGF was mainly localized in the cytoplasm and on the nuclei of hepatocytes, and

to a lesser extent on endothelial and Kupffer cells and some lymphocytes (Figure 2). However, in cirrhotic tissue, it was also markedly expressed on biliary epithelial cells constituting the wall of bile ducts, and on spindle-shaped cells embedded in fibrous tissue (Figure 4). Double staining using anti-NGF and antibody against SMA (Figure 4J and K) indicated that these spindle-shaped cells presumably correspond to the hepatic stellate cells described by other authors[19].

Ultrastructural observation by transmission electron microscopy after immunogold labelling showed that in hepatocytes of HCC tissue (Figure 5), positive reaction for NGF mainly localized on cytoplasmic vesicles (Figure 5B and C) and on endoplasmic reticulum (Figure 5E), suggesting that synthesis and accumulation of this molecule may occur. In some cases, immunogold labelling was also present on nuclei, beneath the nuclear membrane (Figure 5D). Furthermore, in cirrhotic tissue (Figure 6) a more intense immunoreaction, with respect to HCC tissue, was observed on cytoplasmic vesicles (Figure 6B), free in the cytoplasm (Figure 6B) and along endoplasmic reticulum (Figure 6B) of hepatocytes, indicating that in cirrhotic tissue NGF may be actively produced by hepatocytes. Positive immunogold reaction was also observed on cytoplasmic vesicles of endothelial cells (Figure 6C) and of spindle-shaped cells (Figure 6D). In biliary epithelial cells NGF localized in large cytoplasmic vacuoles that were present in the portion of cells near the ductal lumen (Figure 6E).

Expression of TrkA^{NGF} in liver biopsies

Immunohistochemical staining and CLSM observation showed that in HCC tissue trkA^{NGF} localized on the cell membrane of few hepatocytes (Figure 7A$_2$), while in cirrhotic tissue an intense immunoreactivity was also observed on biliary epithelial cells (Figure 7B$_3$ and 7B$_6$). This was particularly evident beneath the cell membrane near the ductal lumen (Figure 7B$_3$), and on spindle-shaped cells scattered throughout fibrotic tissue surrounding reactive bile ducts (Figure 7B$_6$). Positive immunoreaction was also observed on endothelial cells constituting the wall of blood vessels (Figure 7B$_6$).

Furthermore, in cirrhotic tissue, immunogold labelling showed that in biliary epithelial cells (Figure 8A) trkA^{NGF} localized on cell membrane and on cytoplasmic vesicles beneath it (Figure 8A$_1$), suggesting a possible receptor recycling after receptor-ligand reaction. Immunoreactivity was also observed in the cytoplasm of some endothelial cells (Figure 8B$_1$) and on cell membrane of some lymphocytes (Figure 8B$_2$).

NGF detection in sera

NGF levels were measured in sera obtained from 27 patients with documented cirrhosis, HCC, or both by immunoenzymatic assay. All patients disclosed with circulating NGF levels elevated 25-fold over the normal with (range 73-520 pg/mL, compared to a mean of 20 pg/mL in healthy donors, Figure 9).

DISCUSSION

The expression of NGF and its receptors in human and rodent liver cells has been investigated for some time in previous studies with contradictory results. Oakley *et al*[29] demonstrated for example, that hepatocytes expressed NGF, but not trkA^{NGF} during profibrotic liver injury, in early preneoplastic lesions and HCCs, suggesting that in the hepatocytes NGF may not be an autocrine factor but may rather act in a paracrine manner. Other studies[28] reported that in HCC tissue from male B6C3F1 mice, trkA^{NGF} is expressed exclusively in the walls of arteries associated with tumors, presumably in smooth muscle cells, whereas it is negative in other cell types including tumor cells. The same authors also demonstrated that NGF was expressed not only in HCCs but also in early preneoplastic lesions, possibly representing a very early change during mouse hepatic carcinogenesis. On the contrary, Cassiman *et al*[19] provided evidence that in human and rat cirrhotic tissues, NGF and other neurotrophins, as well as their receptors, were expressed not only in hepatic stellate cells, but also in regenerating bile ducts and in some hepatocytes with a cytoplasmic or nuclear staining patterns. Recently it has been reported that NGF is over expressed in approximately 60% of human HCC tissues compared to the surrounding liver tissue with cirrhosis and chronic hepatitis, suggesting a role for NGF in the progression of HCC[32]. Also, until now, to our knowledge, it has not yet been clarified what role NGF plays and which cell types in the liver tissue are actually involved in the NGF mediated cross-talk during liver fibrosis and HCC progression. In addition, even if increased levels of circulating NGF have been reported in several autoimmune, chronic inflammatory and fibrotic disorders[4,5], meanwhile data concerning circulating NGF levels in HCC or cirrhotic patients are not known.

In this study we provide immunohistochemical evidence that NGF and its high-affinity receptor trkA^{NGF} are over expressed in patients suffering from HCC and to a greater extent from HCC with cirrhosis. Surprisingly, NGF and trkA^{NGF} were negative in liver specimens from patients with cirrhosis undergoing transplantation but without HCC, supporting the hypothesis of an active role for NGF in HCC insurgence and progression. The elevated circulating NGF levels recorded in sera from patients with documented cirrhosis, HCC or both, as well as the tissue distribution of NGF and its receptor further support the correlation between NGF activity and the progression of liver fibrosis towards HCC. We also have been able to identify the NGF and trkA^{NGF} immuno positive cell types in liver tissues from cirrhotic and HCC patients, comparing the results obtained by immunohistochemistry and immuno-electron microscopy, as summarized in Table 2.

The evidence that hepatocytes in HCC and cirrhotic tissues from the same liver produce NGF (as suggested by the localization on cytoplasmic vesicles and on endoplasmic reticulum) and express its receptor strongly support the hypothesis that NGF may act by both autocrine and paracrine mechanisms, rather than a prevalent paracrine one, as previously suggested[29].

NGF and trkA^{NGF} have been reported over expressed *in vivo* in proliferating cholangiocytes after bile duct ligation. NGF also stimulates rat cholangiocyte proliferation *in vitro*[33]. We observed that in cirrhotic tissue both NGF and its receptor are over expressed on proliferating biliary

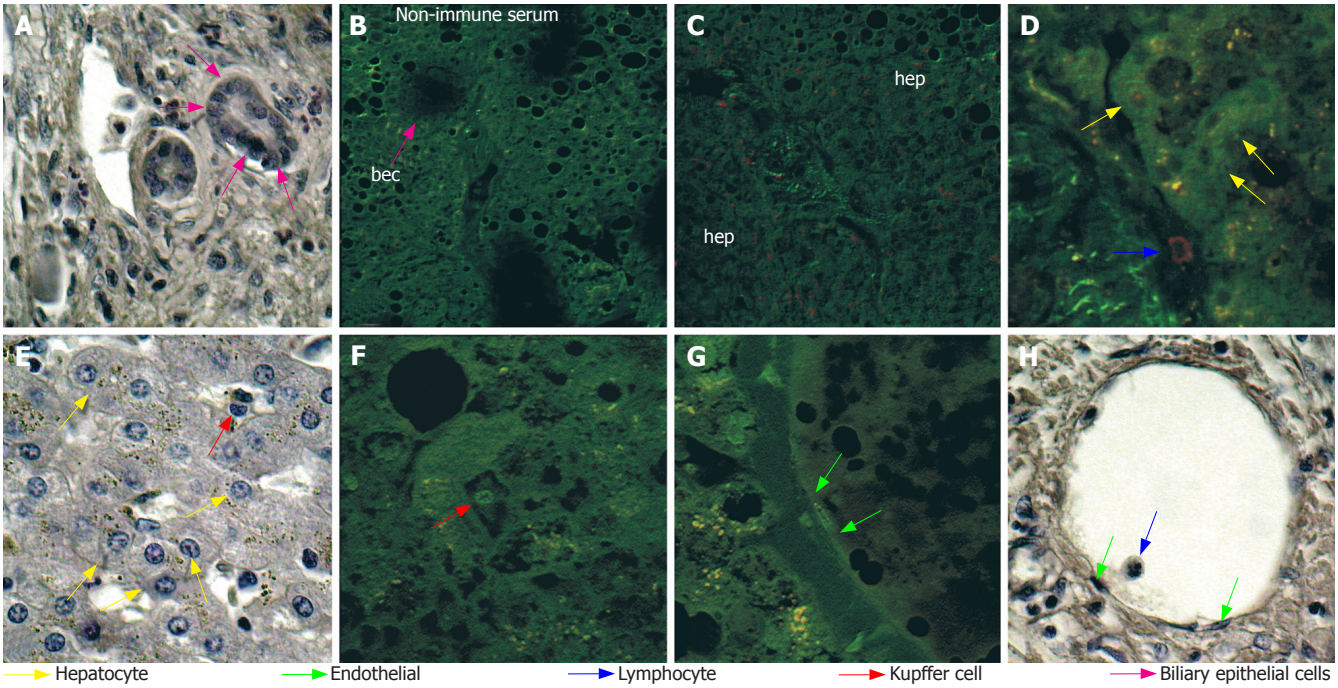

Figure 1 NGF distribution in tissues from healthy donors. **B**, **C**, **D**, **F**, **G**: Confocal microscopy images of immuno-stained sections; in negative hepatocytes, endothelial cells, biliary epithelial cells and Kupffer cells the immunoreaction is the same as the background obtained in control performed by substitution of primary antisera with non-immune rabbit serum; positive immunoreaction (red hue) is only observed on lymphocytes in blood vessels. Green hue represents the auto-fluorescence used to visualize liver tissue morphology; **A**, **E**, **H**: Images of H&E stained sections close to that used for immunohistochemistry. Differently coloured arrows indicate the different cell types (see legend). bec: biliary epithelial cells; hep: hepatocytes.

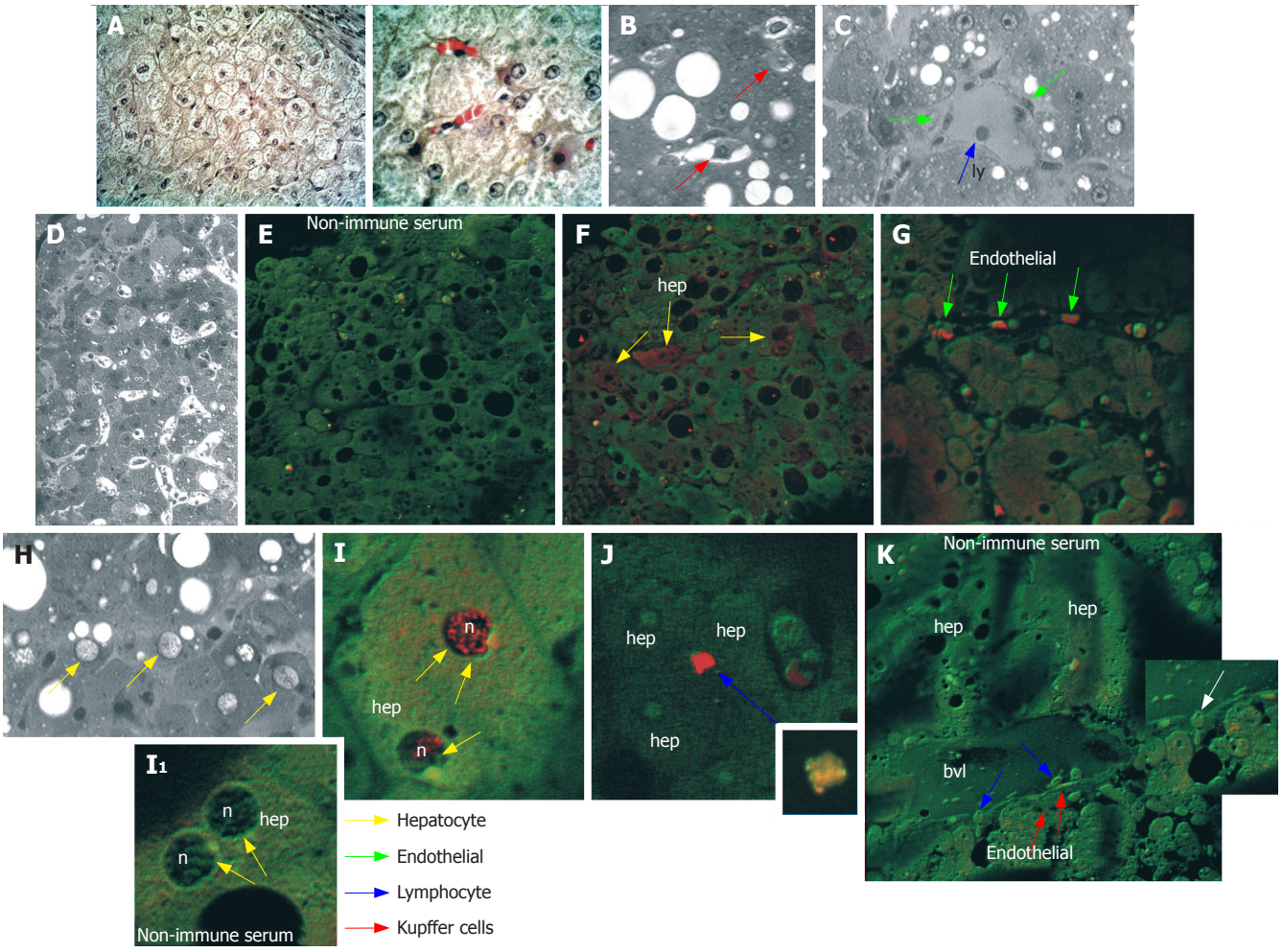

Figure 2 NGF distribution in HCC tissue. **E**, **F**, **G**, **I**, **J**, **K**: Confocal microscopy images of immuno-stained sections showing NGF immunoreaction (red hue) in the cytoplasm (**F**) and on nuclei (**I**) of some hepatocytes, on endothelial cells, on lymphocytes and on Kupffer cells; no immunoreaction was observed in controls performed by substitution of primary antisera with non-immune rabbit serum. **A**: Images, at two different magnification, of H&E stained sections; **B**, **C**, **D**, **H**: Images of semithin sections from samples embedded in Spurr epoxy resin. Differently coloured arrows indicate the different cell types (see legend). n: nucleus; hep: hepatocyte; bvl: blood vessel lumen.

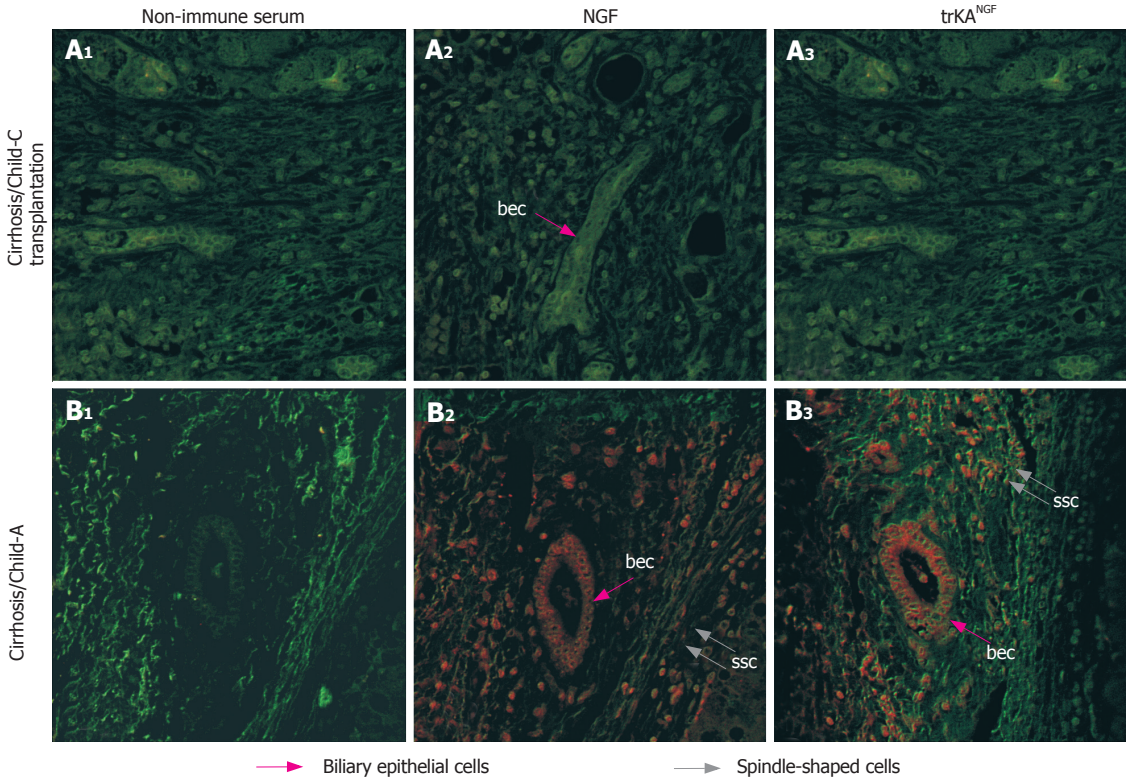

Figure 3 NGF and trkA^{NGF} distribution in cirrhotic tissues by confocal microscopy. **A**$_1$: Liver specimens obtained before transplantation from patients with cirrhosis but without HCC (Child-Pugh C); **A**$_2$: NGF distribution; **A**$_3$: trkA^{NGF} distribution; **B**$_1$: Specimens from patients with cirrhosis, also suffering from HCC, at early staging (Child-Pugh A); **B**$_2$: NGF distribution; **B**$_3$: trkA^{NGF} distribution. NGF or trkA^{NGF} immunoreaction (red hue) is particularly evident on biliary epithelial cells and on spindle-shaped cells, in cirrhotic tissue from patient at early staging (Child-Pugh A), but in presence of HCC, while no immunoreaction is observed in liver specimens resected before transplantation (Child-Pugh C) from patients without HCC. No immunoreaction is observed in controls performed by substitution of primary antisera with non-immune rabbit serum. bec: biliary epithelial cells; ssc: spindle-shaped cells.

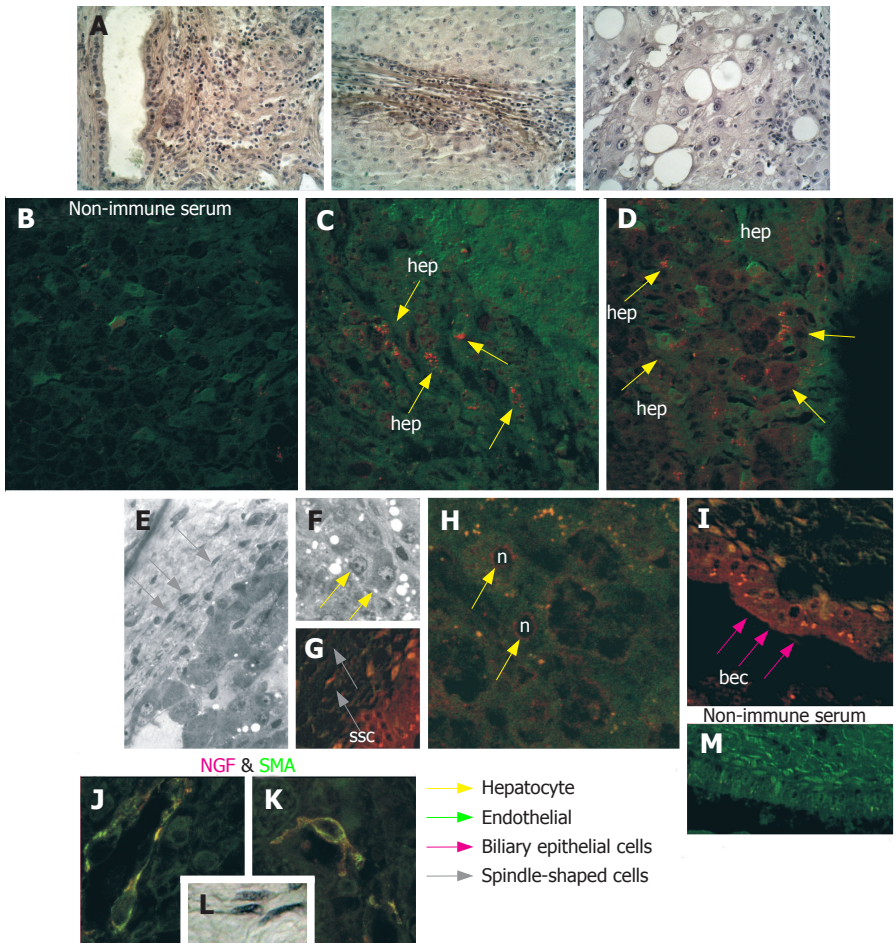

Figure 4 NGF distribution in cirrhotic tissue from patient also suffering from HCC. **B**, **C**, **D**, **G**, **H**, **I**, **M**, **J**, **K**: Confocal microscopy images of immunostained sections showing NGF immunoreaction (red hue) in the cytoplasm of some hepatocytes, on biliary epithelial cells and on spindle-shaped cells; no immunoreaction is observed in controls performed by substitution of primary antisera with non-immune rabbit serum. **J**, **K**: Double staining on spindle-shaped cells using anti-NGF and antibody against the myofibroblast marker smooth muscle actin (SMA); **A**, **L**: Images of H&E staining; **E**, **F**: Images of semithin sections from samples embedded in Spurr epoxy resin. Differently coloured arrows indicate the different cell types (see legend). bec: biliary epithelial cells; n: nucleus; hep: hepatocyte; ssc: spindle-shaped cells.

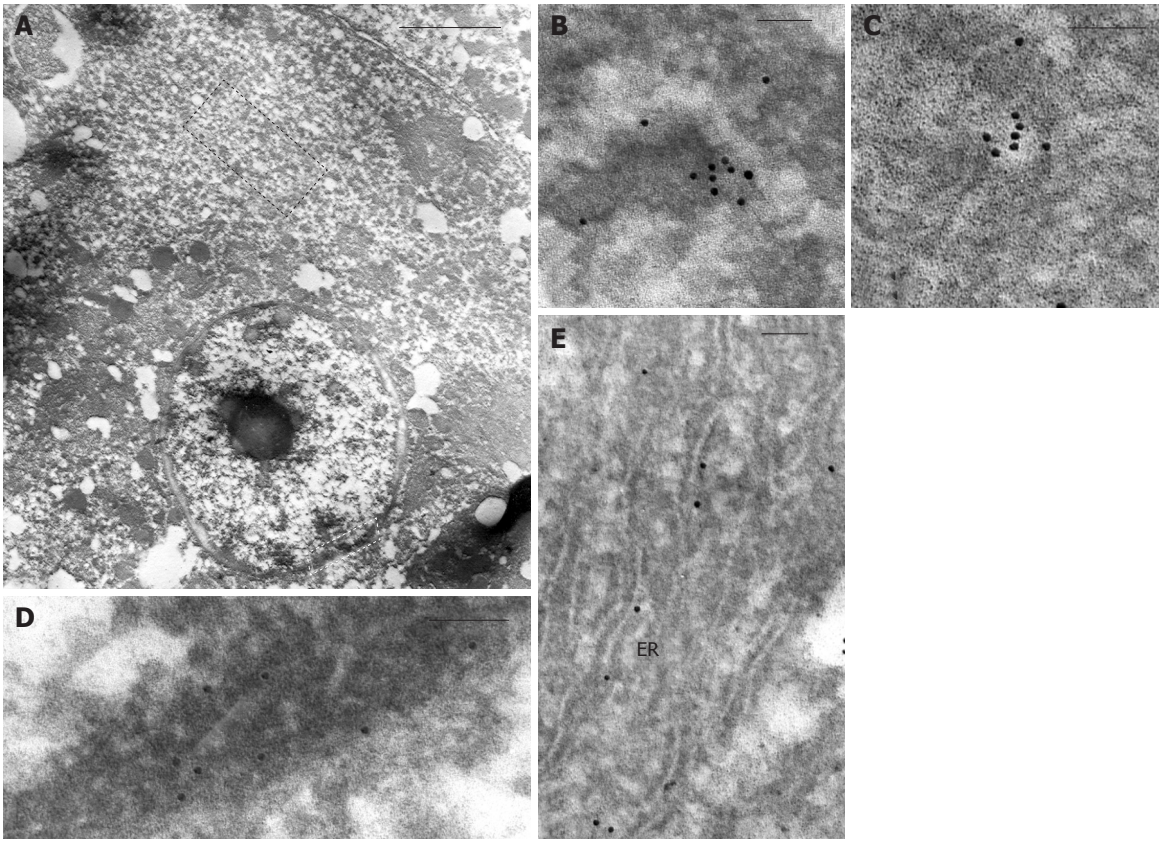

Figure 5 NGF distribution in HCC tissue by immunogold labelling. **A**: Transmission electron micrographs of hepatocyte; **B**, **C**: Positive immunogold reaction on cytoplasmic vesicles; **D**: Positive immunogold reaction on nuclei; **E**: Positive immunogold reaction on endoplasmic reticulum. ER: endoplasmic reticulum. Scale bars = **A**: 2 μm; **B**-**E**: 100 nm.

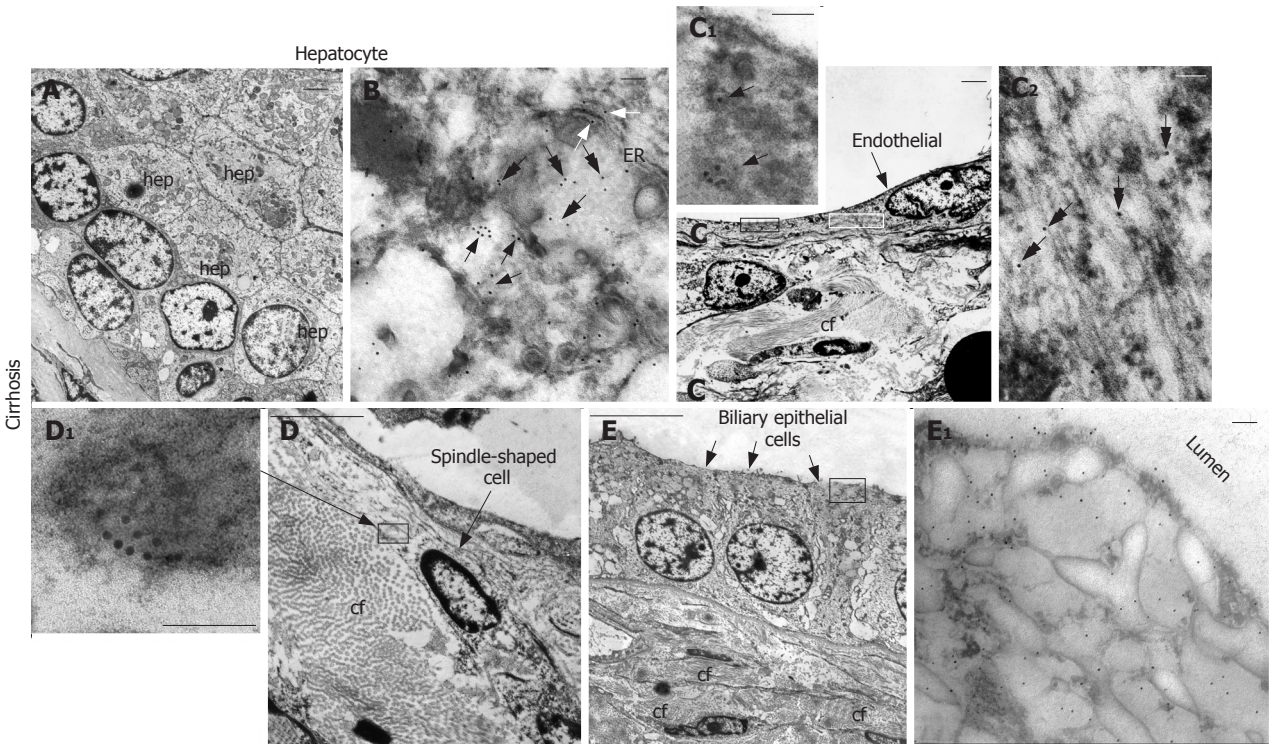

Figure 6 NGF distribution in cirrhotic tissue from patient with HCC by immunogold labelling. **A**, **B**: Transmission electron micrographs of hepatocytes showing positive immunogold reaction on cytoplasmic vesicles (black arrows), free in the cytoplasm (double pointed arrows) and along endoplasmic reticulum (white arrows); **C**, **C**$_1$, **C**$_2$: Endothelial cells; positive immunogold reaction on cytoplasmic vesicles (black arrows) and free in the cytoplasm (double pointed arrows); **D**, **D**$_1$: Spindle-shaped cells; **E**, **D**$_1$: Biliary epithelial cells. In **C**$_1$, **C**$_2$, **D**$_1$ and **E**$_1$ details at higher magnification are shown. hep: hepatocytes; cf: collagen fibers; ER: endoplasmic reticulum. Scale bars = **A**, **C**, **D**, **E**: 2 μm; **B**, **C**$_1$, **C**$_2$, **D**$_1$, **E**$_1$: 100 nm.

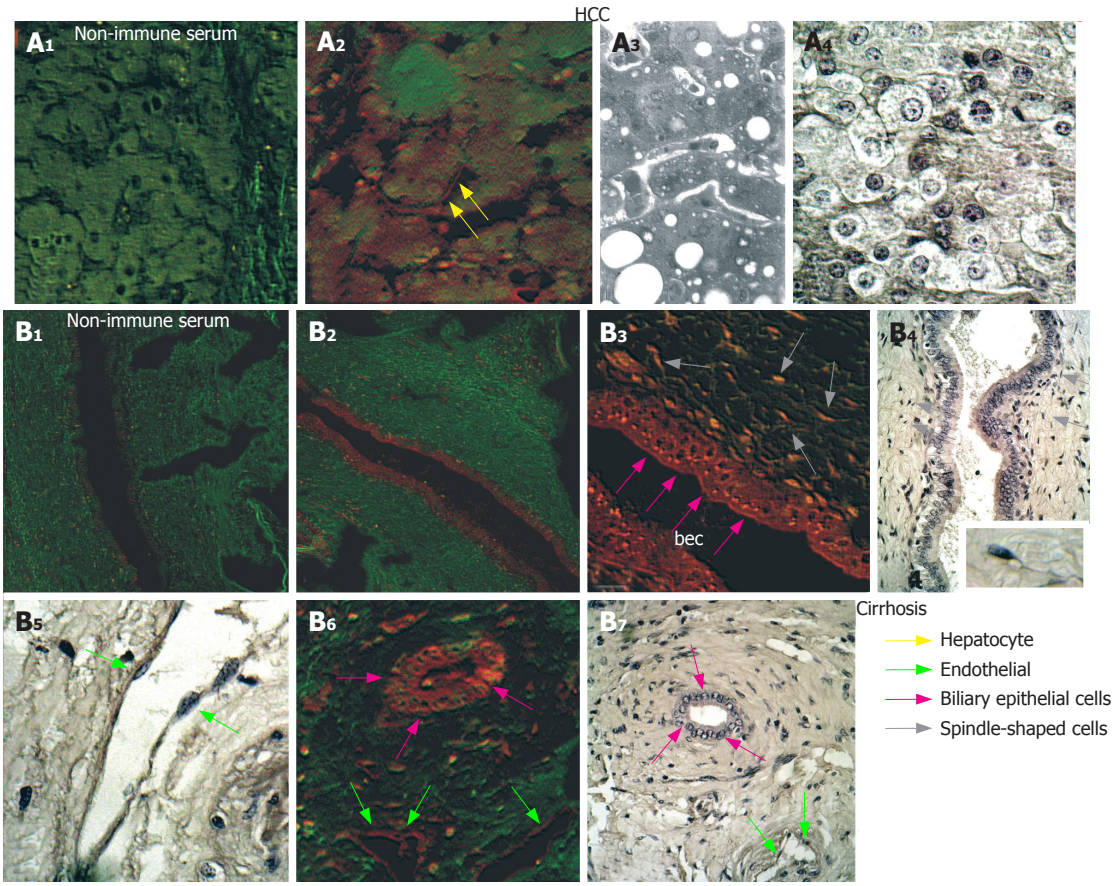

Figure 7 Trk^{ANGF} distribution in HCC and cirrhotic tissues. **A**: HCC tissue; **B**: Cirrhotic tissue. **A**$_1$, **A**$_2$, **B**$_1$, **B**$_2$, **B**$_3$, **B**$_6$: Confocal microscopy images of immuno-stained sections. No immunoreaction was observed in controls performed by substitution of primary antisera with non-immune rabbit serum; **A**$_4$, **B**$_4$, **B**$_5$, **B**$_7$: Images of HE stained sections; **A**$_3$: Images of semithin section from samples embedded in Spurr epoxy resin. Differently coloured arrows indicate the different cell types (see legend). bec: biliary epithelial cells.

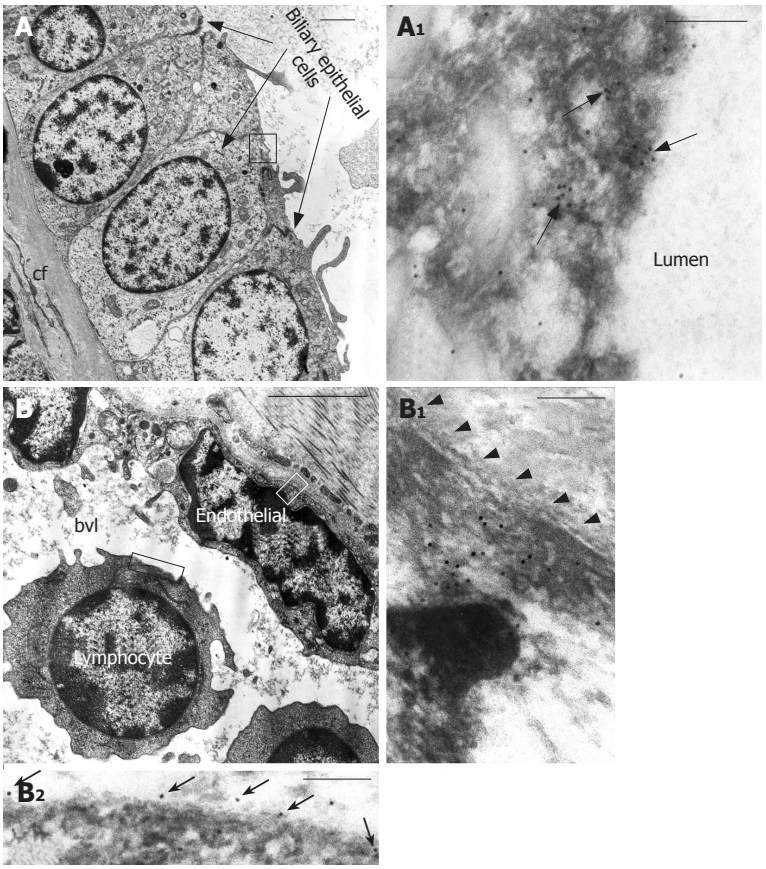

Figure 8 TrkA^{NGF} distribution in cirrhotic tissue from patient with HCC by immunogold labeling. **A**, **A**$_1$: Transmission electron micrographs of biliary epithelial cells showing positive immunogold reaction on cell membrane and cytoplasmic vesicles (black arrows) near the ductal lumen; **B**: Transmission electron micrographs of endothelial cells and lymphocytes; **B**$_1$: Immunoreactivity free in the cytoplasm of endothelial cells beneath the cell membrane (arrowheads); **B**$_2$: Gold particles on cell membrane of a lymphocyte. In **A**$_1$, **B**$_1$ and **B**$_2$ details at higher magnification are shown. bvl: blood vessel lumen; cf: collagen fibers. Scale bars = **A**, **B**: 2 μm; **A**$_1$, **B**$_1$, **B**$_2$: 200 nm.

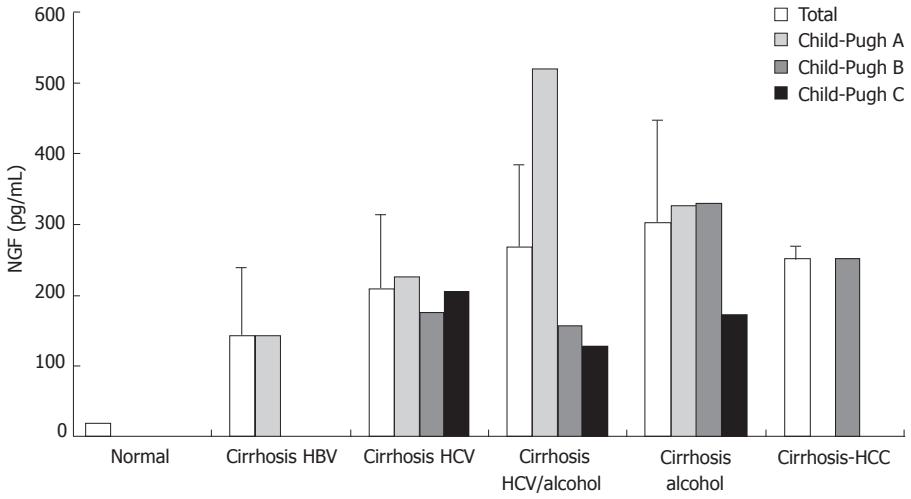

Figure 9 Bar diagram illustrating the circulating NGF levels, determined by test ELISA, in patients with documented cirrhosis/HCC. NGF amounts, reported with regard to the etiology, is calculated either as mean ± SD (all patients examined) or as mean values for Child-Pugh class A (score = 5-6), for Child-Pugh class B (score = 7-9) and for Child-Pugh class C (score = 10-15). As a control, mean ± SD of circulating NGF levels from some healthy individuals is also reported.

Table 2 Expression of NGF and trkA^{NGF} in liver cell types from healthy donors and from patients with cirrhosis and HCC

	Hepatocytes		Biliary epithelial cells		Endothelial cells		Spindle-shaped cells		Lymphocytes		Kupffer cells		Determination methods
Health	NGF	-	NGF	-	NGF	-	NGF	nd	NGF	+	NGF	-	CLSM (IF)
	trkA^{NGF}	-	trkA^{NGF}	-	trkA^{NGF}	-	trkA^{NGF}	nd	trkA^{NGF}	nd	trkA^{NGF}	-	
HCC	NGF	+[1]	NGF	±	NGF	+	NGF	nd	NGF	+	NGF	+	CLSM (IF)
	trkA^{NGF}	±	trkA^{NGF}	±	trkA^{NGF}	±	trkA^{NGF}	nd	trkA^{NGF}	nd	trkA^{NGF}	±	TEM (IG)
CIRR	NGF	++[1]	NGF	++[2]	NGF	++	NGF	+	NGF	+	NGF	±	CLSM (IF)
	trkA^{NGF}	++	trkA^{NGF}	++[2]	trkA^{NGF}	+	trkA^{NGF}	+	trkA^{NGF}	+	trkA^{NGF}	+	TEM (IG)

IF: immunofluorescence labelling; IG: immunogold labelling; nd: not determined. [1]Immunoreaction mainly localized on cytoplasmic vesicles and endoplasmic reticulum. [2]Immunoreaction mainly localized in the portion of cells near the ductal lumen.

epithelial cells, mainly localized on large cytoplasmic vacuoles in the ductal lumen portion of cells suggesting an intraductal lumen secretion during the progression from cirrhosis to HCC. Other studies[34,35] have shown that binding of NGF to its cognate receptor trkA^{NGF} induces formation of signalling endosomes containing both the NGF and activated trkA^{NGF} receptor, with the latter exhibiting very high dynamic trafficking between the cell surface and internal cell compartments. Therefore, the predominant staining on cytoplasmic vacuoles observed for trkA^{NGF} on biliary epithelial cells in cirrhotic tissue may represent the receptor endocellular trafficking. Taken together, these findings indicate that NGF and its related receptor play an important role also in modulating the physiopathology of the intrahepatic biliary epithelium in the course of liver tissue remodelling processes and HCC progression.

The mechanism of NGF involvement in liver tissue remodelling processes and HCC progression remain unclear. However, our observation, defining NGF distribution both inside the liver and in the intracellular compartments, indicate that it may function, in a paracrine and an autocrine manner, as a messenger molecule in the cross-talk between different cell types. This, in addition to the reported lack of NGF expression in cirrhotic tissue without concomitant HCC even if present at high level in serum, strongly support the need to further clarify the mechanism and to define the role of this neurotrophin.

This opens up an interesting perspective for the possible use of NGF, not only as a marker of progression and transformation, but also as an attractive target for a new therapeutic approach.

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