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Heparanase gene expression and its correlation with spontaneous apoptosis in hepatocytes of cirrhotic liver and carcinoma

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Abstract

Heparanase (hep) degrades heparan sulphate proteoglycans (HSPGs), which are the main components of the extracellular matrix. This process has been considered as the first step of tumour invasion or metastasis. However, HSPGs play an important role in signal transduction. Thus, the degradation of HSPGs by hep may suppress tumour cell growth. In the present study, we investigated the clinicopathological importance of enhanced *hep* mRNA expression in 48 hepatocellular carcinomas (HCCs) and in 48 non-cancerous liver samples obtained from the same patients by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Spontaneous apoptosis in the hepatocytes was evaluated by immunohistochemistry. The relative *hep* mRNA expression levels were described as *hep*/glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) ratios. The *hep* mRNA levels of HCCs were significantly lower than those of non-cancerous livers ($P<0.001$). *Hep* mRNA levels decreased with increasing liver fibrosis. A significant positive correlation between hep gene expression and spontaneous apoptosis was detected. *Hep* expression in the tumours did not correlate with tumour differentiation or with tumour stage. However, low *hep* gene expression was associated with a poor disease-free survival of the patients. Thus, *hep* gene expression may play an important role in programmed cell death and this gene expression may be lost during the malignant transformation of hepatocytes.

Keywords: Apoptosis; Heparanase; Heparan sulphate proteoglycans; Hepatocellular carcinoma; Real-time reverse transcriptase polymerase chain reaction

1. Introduction

Heparan sulphate proteoglycans (HSPGs) present at the cell surface and in the extracellular matrix around cells. Basic fibroblast growth factor (bFGF) acts in cell growth and development [1]. The biological activity of bFGF is mediated by an interaction with transmembrane receptor tyrosine kinases (RTKs) [2]. bFGF binds to HSPGs on the cell surface. Stable binding of bFGF to RTKs and signalling requires the presence of HSPGs [3,4]. This ternary complex of bFGF/HSPGs/RTKs plays an important role in malignant tumour progression [5].

Heparanase (hep) is an endoglycosidase that degrades the heparan sulphate chain of HSPGs. Recently, protein or messenger RNA (mRNA) expression of hep has been identified in various cancer cells, and the overexpression of hep protein or mRNA in tumour cells has been reported to correlate with the metastatic potential of tumour cells *in vitro* and *in vivo* [6,7] as well as with poor patient prognosis [8,9]. However, hep mRNA or protein overexpression may destroy the cell-surface bFGF/HSPGs/RTKs complex and may downregulate tumour cell growth signals. Thus, the role of hep in malignant tumours remain unclear. In the present study, to evaluate the clinicopathological significance of *hep* gene expression in hepatocellular carcinoma (HCC), we analysed *hep* mRNA expression levels in carcinoma and non-cancerous liver tissue using the real-time reverse transcriptase polymerase chain reaction (RT-PCR) method.

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2. Patients and methods

2.1. mRNA extraction from tissue

We obtained tumours and non-cancerous liver tissues from 48 patients with HCC. These 48 patients underwent hepatectomies between 1993 and 1999. Informed consent was obtained from all patients for the subsequent use of their resected tissues. The present study conformed to the ethical standards of the World Medical Association Declaration of Helsinki. Tissue samples of approximately 1 g were collected immediately after liver resection. Non-cancerous liver tissues were obtained from regions distant from the tumours. Half of the tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm thick) were prepared for haematoxylin-eosin staining for histopathological diagnosis and for immunohistochemical staining. The other half of the tissue was stored at −80 °C until needed. Before starting the study, histopathological examination confirmed that no cancer cells had contaminated the non-cancerous liver tissues. Total RNA from tissues was isolated using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesised with 1 μg of total RNA with Ready-to-Go™ You-Prime First-Strand Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The final volume was adjusted to 50 μl with diethylpyrocarbonate (DEPC) water.

2.2. Real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay

The following primers and TaqMan probes were used for *hep*, forward primer 5′-TCACCATTGACGCCAACCT-3′, reverse primer 5′-CTTTGCAGAACCCAGGAGGAT-3′, and probe 5′-FAM (6-carboxy-fluorescein)-CCACGGACCCGCGGTTCCT-3′-TAMRA (6-carboxy-tetramethyl-rhodamine) [10]; and for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), forward primer 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse primer 5′-GAAGATGGTGATGGGATTTC-3′, and probe 5′-FAM-CAAGCTTCCCGTTCTCAGCC-3′-TAMRA [11]. AmpliTaq DNA polymerase extended the primer and displaced the TaqMan probe through its 5′- 3′ exonuclease activity. Quantification of gene expression was performed using real-time quantitative RT-PCR (Gene Amp® 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA)), which uses the 5′ nuclease activity of Taq polymerase to detect PCR amplicons [12,13]. The PCR solution (50 μl) was composed of 1 μl of cDNA, 5 pmol of the forward and reverse primers, 10 pmol of internal probe, and TaqMan Universal PCR Master Mix. PCR was carried out after incubation at 50 °C for 2 min and denaturing at 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 61 °C for 1 min. The threshold line was set at a Rn (normalised reporter signal) of 0.05 [12]. The point at which the amplification plot crossed this threshold was defined as Ct, which represented the cycle number at this point. Standard curves for *GAPDH* and *hep* were generated using serial dilution (containing 1000, 200, 40, and 8 ng) of total RNA derived from HepG2 (HCC cell line, Riken Gene Bank, Tsukuba Science City, Japan). The plots represent the log of the input amount (log ng of total starting RNA) as the *x*-axis and Ct as the *y*-axis. Equations were derived from the lines of the calibration curves [12]. The two formulas for log ng of *hep* and *GAPDH* were as follows: *hep*, $y = 42.4–5.2x$-($r^2 = 0.97$); *GAPDH*, $y = 31.2–3.5x$-($r^2 = 0.97$). For each sample, the amount of *hep* and *GAPDH* mRNAs was determined from the standard curves. *GAPDH* transcripts were monitored as a control to quantify the transcripts of the genes in each sample. The relative *hep* mRNA levels of tissues were normalised by *hep*/*GAPDH* mRNA ratios in each sample.

2.3. Immunohistochemistry

To detect apoptotic cells, a polyclonal rabbit anti-single-stranded DNA (ssDNA; diluted 1:200; DAKO Japan Co., Ltd., Kyoto, Japan) was used as a primary antibody [14]. Between 1000 and 2000 cells were examined by one observer. The results were expressed as the apoptotic index (AI; percentage of immunostained cells).

2.4. Patients

The subjects included 38 men and 10 women, and their average age at the time of surgery was 61.6 years (ranging from 35 to 80 years). Histopathological diagnoses of the patients were made according to the guidelines for the classification of primary liver cancer [15]. None of the patients had received preoperative or postoperative chemotherapy. All patients were followed up until December 2001 with a mean follow-up period of 44.6 months (ranging from 2 to 97 months). Recurrence of HCC was detected by computed tomography and ultrasonography.

2.5. Statistics

All real-time RT-PCR analyses were repeated three times and the mean value of *hep*/*GAPDH* mRNA ratio was used. The *hep*/*GAPDH* mRNA ratios for the different clinicopathological parameters were evaluated by Mann–Whitney's U-test and Kruskal–Wallis test. The relationship between *hep*/*GAPDH* mRNA ratios and AIs in liver tissues was evaluated statistically using the

Spearman's rank correlation test. Disease-free survival rates were calculated using the Kaplan–Meier method. The log rank test was used for comparisons of the two survival curves. P values of less than 0.05 were considered statistically significant.

3. Results

The results from patient samples were plotted on the standard curve. The estimated amounts of *hep* and *GAPDH* mRNA were calculated. The *hep*/*GAPDH* ratios indicated relative *hep* gene expression in each sample. The mean *hep*/*GAPDH* mRNA ratio of the 48 tumours was 2.9 (standard deviation: 4.1, range: 0.2–18.3, median: 1.0) and that of 48 non-cancerous liver tissues was 5.6 (standard deviation: 4.6, range: 0.6–17.2, median: 4.2). The average *hep*/*GAPDH* mRNA ratio of the non-cancerous liver tissues was significantly higher than that of the HCCs ($P<0.001$). The *hep*/*GAPDH* mRNA ratio did not correlate with tumour differentiation or with tumour stage (Table 1). However, in the 48 non-cancerous liver tissues, the mean *hep*/*GAPDH* mRNA ratio of 33 liver tissues with severe liver fibrosis (4.5 ± 3.4) was lower than that of 15 liver tissues with mild liver fibrosis (7.8 ± 5.8, $P=0.047$).

The mean AI of 48 non-cancerous liver tissues (3.5%, range: 0–22.8%) was significantly higher than that of the 48 tumours (1.6%, range: 0–9.3%, $P=0.011$). Fig. 1 shows the apoptotic hepatocytes in the non-cancerous liver tissue. The mean AIs of well differentiated carcinoma ($n=6$, 1.5%), moderately ($n=32$, 1.3%), and poorly ($n=10$, 2.8%) were not significantly different ($P=0.443$). In addition, the mean AIs of the five Stage I tumours (1.2%), 12 Stage II tumours (1.7%), 21 Stage III tumours (2%), and 10 Stage IV tumours (1%) were not different ($P=0.847$). A significant positive correlation ($\rho=0.667$, $P<0.001$) between *hep*/*GAPDH* mRNA ratios and AIs was detected in 96 liver tissues (both tumours and non-cancerous liver tissues) (Fig. 2).

Up to the end of December 2001, 30 of 48 patients (63%) had been diagnosed as having recurrent tumours in their residual livers. The 48 patients were divided into two subgroups (high *hep*/*GAPDH* mRNA ratio >1.0, $n=23$ and low *hep*/*GAPDH* mRNA ratio $\leqslant1.0$, $n=25$) according to the median *hep*/*GAPDH* mRNA ratio of the 48 tumours. The disease-free 5-year survival rate of the 23 patients with high *hep* mRNA expression in their tumours (57.8%) was significantly better than that of the 25 patients with low *hep* mRNA expression (19.8%, $P=0.018$).

4. Discussion

HSPGs are complex glycosaminoglycan consisting of polysaccharide chains of up to 400 modified sugar residues in length. HSPGs are essential components of the extracellular matrices. Invasion into the surrounding

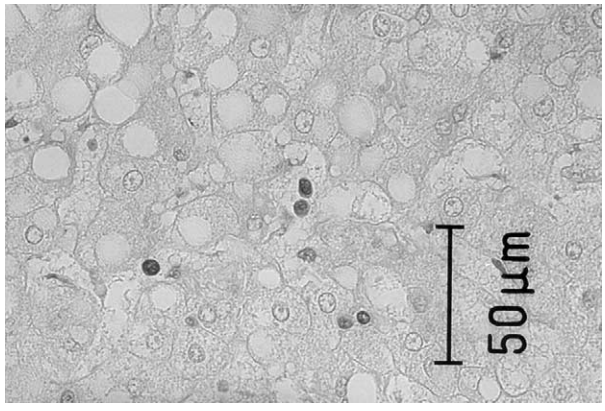

Fig. 1. Apoptotic hepatocytes in non-cancerous liver tissue detected by polyclonal rabbit anti-single-stranded DNA (×100).

Table 1
Correlation between clinicopathological parameters and *hep*/*GAPDH* mRNA ratios in tumours

Parameters	*N*	*hep*/*GAPDH* mRNA ratio (mean±S.D.)	*P* value
Histological type			
Well	6	2.3±2.9	
Moderate	32	3.2±4.6	0.488
Poor	10	2.0±3.0	
Tumour stage			
I	5	5.8±6.4	
II	12	2.3±2.3	0.457
III	21	3.3±4.9	
IV	10	1.1±1.1	

P values were evaluated by the Kruskal–Wallis test. S.D., standard deviation.

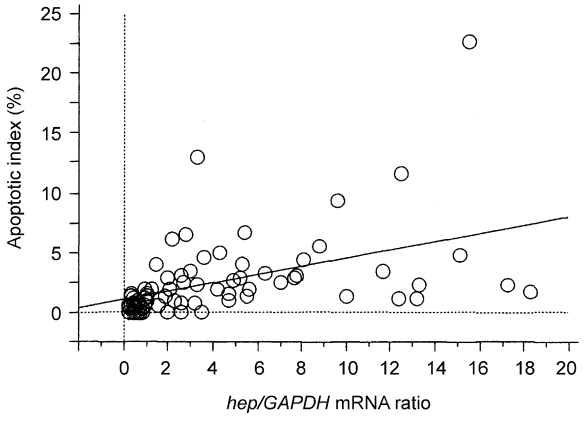

Fig. 2. Correlation between relative heparanase (*hep*) mRNA expression levels (x axis) and apoptotic indices (%, y axis) in liver tissues (tumours and non-cancerous liver tissues). A significant positive correlation ($\rho=0.667$, $P<0.001$) was detected.

matrix is a fundamental characteristic of cancer cells. To degrade extracellular HSPGs, cells express the enzyme hep, an endoglycosidase that cleaves HSPGs chains at a limited number of sites along the polysaccharide chain [16]. Hep activity has been reported to correlate with the metastatic potential of tumour cells in an animal model [7]. In human cancers, Ikuta and colleagues [17] have reported that the incidence of metastasis in oral cancer patients correlates with a high *hep* mRNA expression level of tumours. In addition, Koliopanos and colleagues [10] have reported a significant correlation between enhanced *hep* mRNA expression and decreased postoperative survival in pancreatic cancer. These observations strongly suggest that enhanced *hep* mRNA expression correlates with the metastatic potential of tumours.

However, our results for HCC are completely the opposite to those previously reported. In the present study, we found that the relative *hep* mRNA expression level in HCC is significantly lower than that in the noncancerous liver tissue, and found that tumour *hep* mRNA expression did not correlate with tumour stage. Moreover, we found that *hep* mRNA expression levels were reduced with increasing of liver fibrosis in the noncancerous liver tissues. These findings indicate that *hep* mRNA expression may be lost during the malignant transformation of hepatocytes. Additionally, this reduction in hep mRNA expression in HCC may not correlate with tumour progression. Ogawa and colleagues [18] have established rat HCC cell lines with a high metastatic potential and have found decreased *hep* mRNA expression in one cell line showing high levels of lung metastasis when injected subcutaneously (s.c.) in nude mice. These negative results indicate that *hep* mRNA expression may act as a suppressor in tumour progression or metastasis in HCC.

A traditional RT-PCR method has been used to evaluate *hep* mRNA expression levels [19]. The RT-PCR assay is an easy method for detecting mRNA expression in tissue. The precise amount of total RNA added to each reaction mix is difficult to assess, various housekeeping genes (*GAPDH*, *β-glucuronidase*, or *TATA box-binding* gene) are used as internal controls [12,20,21]. However, the expression levels of internal control mRNAs have been reported to be different among samples using real-time RT-PCR, even if the same amounts of total RNA are used [20–22]. RNA degradation during extraction of RNA from tissues or during the PCR procedure may explain this phenomenon. This difference in the expression levels of housekeeping genes might be overlooked in a Northern blot or a traditional RT-PCR assay. Thus, the results obtained from a RT-PCR method may not reveal true tissue *hep* mRNA levels. In the present study, we used a quantitative real-time RT-PCR method to evaluate the relative expression levels of *hep* mRNA in HCCs. Among the various internal controls, *GAPDH* is the most frequently used, thus to normalise the expression levels of the target gene (*hep*), we used *GAPDH* as a housekeeping gene. At first, we diluted the RNA extracted from the HepG2 cells. Then, we drew standard curves of *hep* mRNA and *GAPDH* mRNA by real-time RT-PCR. For each experimental sample, the amount of *hep* mRNA and *GAPDH* mRNA was determined from the standard curves. Then, the target amount was divided by the endogeneous reference amount to obtain a normalised target value. Thus, even if the expression levels of target and housekeeping gene are varied in individual samples, the real relative target gene expression levels in each sample could be obtained. To avoid amplification of any genomic DNA, the forward and reverse primers for each gene were chosen from different exons. The normalised amount of *hep* gene expression was determined by dividing the amount of *hep* mRNA by the amount of *GAPDH* mRNA for each sample. This method allows us to evaluate more accurately the *hep* mRNA expression levels in tissues than with the RT-PCR method.

Recently, the biological importance of HSPGs has been recognised. Cell-surface HSPGs bind to bFGF and its receptors. This ternary complex plays an important role in signal transduction [5]. Kuniyasu and colleagues [23] have found that heparan sulphate treatment increases the invasive activity of colon cancer cell lines. In addition, Liu and colleagues [24] have reported that treatment with heparinase III, which cleaves at the under-sulphated regions of HSPGs, reduces tumour volume and the number of lung metastases in mice injected with B16BL6 melanoma cells. These findings indicate that HSPGs may play an important role in tumour progression or metastasis. The overexpressed hep protein may therefore destroy this HSPGs/bFGF/receptor complex and, tumour-cell progression may be suppressed.

Surprisingly, high concentrations of hep protein have been detected in apoptotic cells in ovarian cancer by immunostaining [25]. They concluded that apoptosis may be the main mechanism of releasing hep from the cell into the extracellular space. However, we found a significant positive correlation between *hep* mRNA expression levels and the percentages of apoptotic hepatocytes in liver tissues, which indicates a direct interaction between *hep* mRNA expression and the occurrence of apoptosis in the cells. It was reported that B16BL6 melanoma cells injected s.c. in mice, showed a significant increase in apoptosis after heparinase III treatment [24]. This phenomenon suggests that disruption of the HSPGs/bFGF/receptor complex by hep may induce a reduction in the growth signal through the bFGF pathway, leading to apoptosis in these cells. Thus, hep may control growth-signal transduction, and *hep* mRNA expression may be essential for normal cell

turnover. As such, reduced *hep* mRNA expression may result in an abnormal cell growth and may correlate with hepatocarcinogenesis.

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