domain (Fig. 1A). Although all three domains of CAP were conserved, homologies in the C-terminal actin-binding domain were higher than other regions. By immunohistochemical analysis, CAP2 expression could be observed in 24 h post-fertilization (hpf) embryo, and continued in the larva and adult zebrafish. In the early embryo development (Fig. 1B), CAP2 was seen in the neural tube (nt), and a group of tissues that will give rise to a muscle segment, the myotome (my). As the embryo developed to larva, CAP2 expression was observed in the brain (br), and skeletal muscle (sk). In adult zebrafish tissue, CAP2 expression was also observed in the brain, heart and skeletal muscle (Fig. 1C and D). We also compared the expression of CAP2 and CAP1, a homolog of CAP2 that also has been identified in zebrafish [24,25]. Differential expression of CAP2 was particularly observed in the heart and skeletal muscle, where CAP2 was highly expressed (Supplementary Fig. 1A).

CAP2 zebrafish morphants showed an abnormal short-body phenotype

To study CAP2 function in zebrafish, we created CAP2 knockdown morphants by injecting CAP2 antisense morpholino oligonucleotides into cytoplasmic streaming of 1- to 8-cell stage zebrafish embryos. To assess specificity, two non-overlapping morpholinos (A and B) were designed against a CAP2 sequence, with five-mispair control oligo for each morpholino as a negative

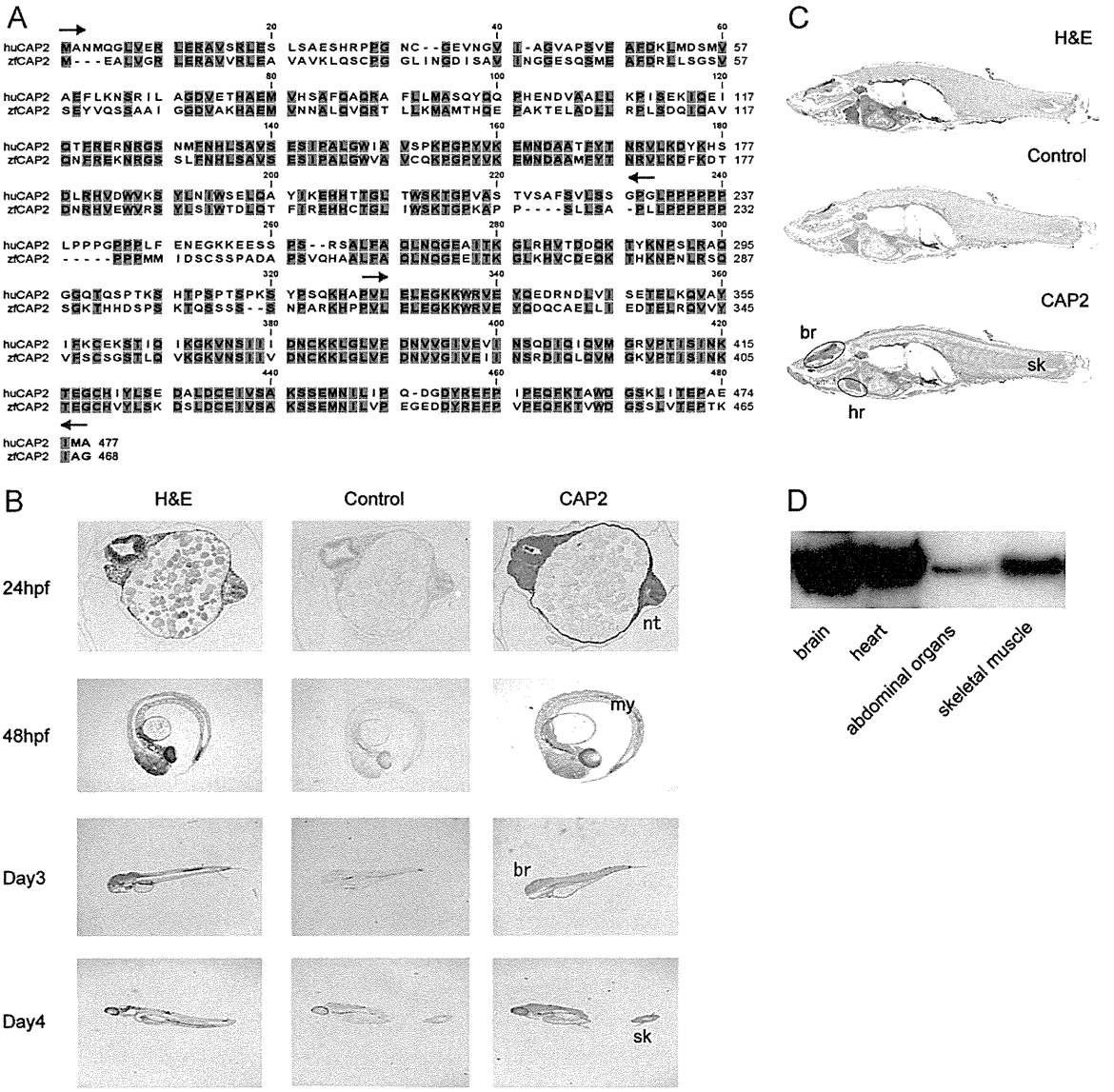

Fig. 1 – Cyclase-Associated Protein 2 (CAP2) is expressed in both embryo and adult zebrafish. (A) Direct amino acid comparison between human and zebrafish CAP2 sequences, with conserved amino acids highlighted in red. The N-terminal domain is depicted by black arrows, C-terminal domain is depicted by red arrows, and between them is a proline-rich sequence. (B) CAP2 expression in zebrafish embryo, 24 and 48 hpf, followed by day 3 and day 4 larva. CAP2 was expressed in the neural tube (nt) and in the myotome (my) of a 48 hpf embryo. In day 3 and day 4 larva, CAP2 was expressed in the brain (br) and skeletal muscle (sk). (C) CAP2 expression in adult male zebrafish was observed in the brain, heart (hr) and skeletal muscle. Negative control was included in each staining. (D) Protein expression was confirmed with Western blot analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

control. Injecting either CAP2 morpholinos resulted in reproducible and identical morphants phenotypes. In 24 hpf, we observed that most of the morphants embryos (MO A, B) showed shorter body length compared with the mispaired control (MP A, B) (Fig. 2A). Additional controls using tracer injected embryos (PR) as well as wild-type embryos (WT) did not show an abnormal phenotype (Fig. 2A). CAP2 morphant embryos also displayed pericardial edema with persistently short-body phenotype at three days post-fertilization, while the mispair control embryos developed similarly to the wild-type (Fig. 2B). To make sure that the experiment is being performed in the effective and specific concentration range, injection of the CAP2 targeted morpholino was performed side-by-side with the five-mispair control oligo, and the resulting short-body phenotype was observed in a concentration-dependent manner (Table 1). At least two independent injection experiments were replicated. Reduced CAP2 expression was observed in CAP2 knockdown morphant embryos with immunohistochemical analysis (Fig. 2C). The morphants were mostly non-motile and did not survive due to stalled development. Additional control using 0.3 mM standard control oligo which has been known can be extensively used without triggering off-target effects showed that the resulting short-body phenotype could be reduced until 17% (Supplementary Table 1). Since CAP1 was also ubiquitously expressed in zebrafish, we performed double knockdown experiments by mixing both of the two sequences of CAP2 and CAP1 morpholinos. Similar short-body phenotype was observed, and a clear reduction of both CAP2 and CAP1 expression was obtained in Western blot analysis (Supplementary Fig. 1B). The number of morphant short-body embryos was not enhanced by the double knockdown strategy (Table 1), suggesting no interference between each other.

CAP2 colocalized with actin both in zebrafish and HCC cell lines

Both CAP2 and CAP1 protein were detected from the zebrafish larval period, however, clear strong expression of CAP2 was particularly observed in the skeletal muscle area, while only thin weak expression of CAP1 was observed in the same area of skeletal muscle. Immunostaining using antibody against muscle actin and alpha-sarcomeric actin, i.e., it only reacts with skeletal muscle actin, also showed a strong clear expression of actin in the zebrafish skeletal muscle area, where CAP2 was also expressed. They both showed a similar expression pattern suggesting colocalization of CAP2 and actin in the skeletal muscle of zebrafish (Fig. 3A, Supplementary Fig. 1C). These findings indicate the possibility that the abnormal short-body phenotype seen in the CAP2 zebrafish morphants may be due to the downregulation of actin-associated CAP2. We also investigated colocalization of CAP2 and actin in human cancer cells. According to our previous report, CAP2 was highly expressed in multistage hepatocarcinogenesis, and we frequently found that the tumor cells invading the stroma were clearly stained with CAP2 [14]. In the present study, we found that CAP2 was highly distributed in the perinuclear area, and was also co-localized with actin in the leading edge of lamellipodium in PLC/PRF/5, and

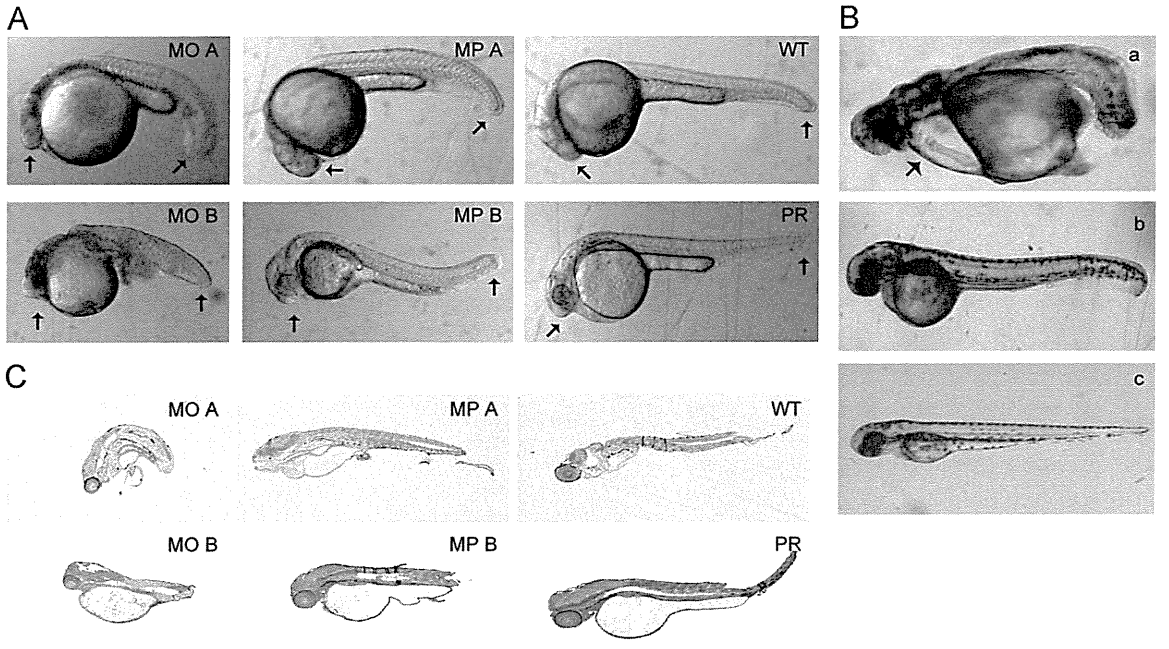

Fig. 2 – Cyclase-associated protein 2 (CAP2) zebrafish morphants showed a short-body phenotype. (A) Shorter body length was observed in morphant-A and morphant-B CAP2 embryos (MO A, MO B) compared with each five-mispair negative control (MP A, MP B), wild-type control (WT), and additional injection controls using embryos injected only with the tracer, phenol red (PR). Black arrows mark the length boundary. (B) The resulting short-body phenotype was persistently observed with pericardial edema (red arrow) at 3 days post fertilization (a), while developmental defects were not observed in morphant controls (b) or wild-type embryos (c). All morphants were injected with 0.3 mM concentration of morpholino. (C) At 5 days post fertilization, immunohistochemical analysis showed reduced expression of CAP2 in both morphants, compared with the mispair controls and wild-type or tracer control embryos. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1 – Concentration-dependence effect of cyclase-associated protein2 (CAP2) morpholinos on early development of zebrafish embryos.

Injections	Embryos injected	Eggs left after 24 h	Normal	Short-body	Percentage showing phenotype
0.1 mM (MO-A)[a]	59	52	41	11	21
0.1 mM (MP-A)[b]	68	61	56	5	8
0.3 mM (MO-A)	154	78	12	66	85
0.3 mM (MP-A)	94	37	25	12	32
0.5 mM (MO-A)	82	35	3	32	91
0.5 mM (MP-A)	86	41	26	15	37
1 mM (MO-A)	118	10	3	7	70
1 mM (MP-A)	120	55	15	40	73
0.3 mM (MO-B)[c]	68	60	3	57	95
0.3 mM (MP-B)[d]	69	62	55	7	11
CAP2A+ CAP1 (MO)	59	26	2	24	92
CAP2A+CAP1 (MP)	83	54	19	35	65
CAP2B+CAP1 (MO)	75	56	7	49	88
CAP2B+CAP1 (MP)	76	54	39	15	28

[a] Antisense morpholino A.
[b] Mispaired control A.
[c] Antisense morpholino B.
[d] Mispaired control B.

the highly metastatic, KYN-2, HCC cell lines (Fig. 3B, Supplementary Fig. 2). These findings suggest that the role of CAP2 in inducing stromal invasion may relate to its actin cytoskeletal function.

CAP2 silencing in HCC cell lines resulted in a defect in lamellipodium formation and a reduction in cell motility

We further assessed the role of CAP2 in relation to its cytoskeletal function. CAP2 knockdown was performed in HCC cell lines using two small interfering RNA molecules (siCAP2 A and siCAP2B). The knockdown effect was confirmed using Western blot analysis (Fig. 4A), and the transfected cells were serum starved for 24 h before stimulation with serum the next day. Within 15 min after serum stimulation, control cells started to spread out and extended lamellipodium around the circumference of the cell clusters. Lamellipodium formation in CAP2-knockdown cells was, however, still restrained or only partial (Fig. 4B). The role of CAP2 in cell motility was evaluated by migration assay with CAP2-knockdown cells. The number of migrating CAP2-knockdown cells decreased compared with the controls (Fig. 4C). We did not observe reduced cell proliferation in CAP2-knockdown cells (data not shown), suggesting that the decreased number of migrated cells was solely due to the reduced cell motility caused by CAP2 knockdown.

CAP2 expression in HCC clinical cases correlated with tumor size, poor differentiation, portal vein invasion, and intrahepatic metastasis

We evaluated CAP2 expression in 105 HCC nodules (21 well differentiated (including 9 early), 68 moderately differentiated, and 16 poorly differentiated HCCs). Immunohistochemical evaluation was done by combining the intensity and positivity of CAP2 staining (Supplementary Fig. 3). Strongly positive expression of CAP2 was mostly observed in progressed HCC. A 2+ score was observed in 10 of the 16 (63%) poorly differentiated HCCs, 30 of the 68 (44%) moderately differentiated HCCs, and 2 of the 21 (10%) well differentiated HCCs (none of the early nodules had 2+ score). Reversely, negative expression was observed in only 2 of the 16 (13%) poorly differentiated HCCs, 3 of the 68 (4%) moderately differentiated HCCs, and 10 of the 21 (48%) well differentiated HCCs (including 6 of the 9 early nodules (67%)) (Table 2). CAP2 expression was significantly associated with tumor size ($P=0.004$), poor differentiation ($P=0.001$), portal vein invasion ($P=0.001$), and intrahepatic metastasis ($P=0.002$) (Table 3).

Discussion

Cyclase-associated protein, CAP, has been identified as a bifunctional protein from yeast through mammals, indicating the retention of important conserved functions during evolution. A homolog of yeast CAP, which is required for proper genesis of cell polarity in eukaryotes, has been identified in *Drosophila*. Loss of *Drosophila* CAP causes cell polarity defects, altering the distribution of actin filaments and resulting in various developmental defects [26]. CAP knockout mutant *Dictyostelium* cells showed changes in cell polarity, F-actin organization, and phototaxis suggesting that CAP may play a critical part in cell polarity and movement of diverse organisms [27]. In human and adult rat tissue, at least two different homologs of CAP, CAP1 and CAP2, have been found, and they share more than 60% amino acid identity [5,6,8]. However, the functions of CAPs in mammals or higher vertebrates are not well established, and in particular, studies on CAP2 are lacking. In the zebrafish, the gene encoding CAP1 has been reported and shares 63% identity with human CAP1 [24]. In the present study, we identified a CAP2 protein which shares 60% identity with human CAP2. Although both CAPs are conserved in zebrafish, their expression pattern is different. We observed that CAP2 was expressed in the brain,

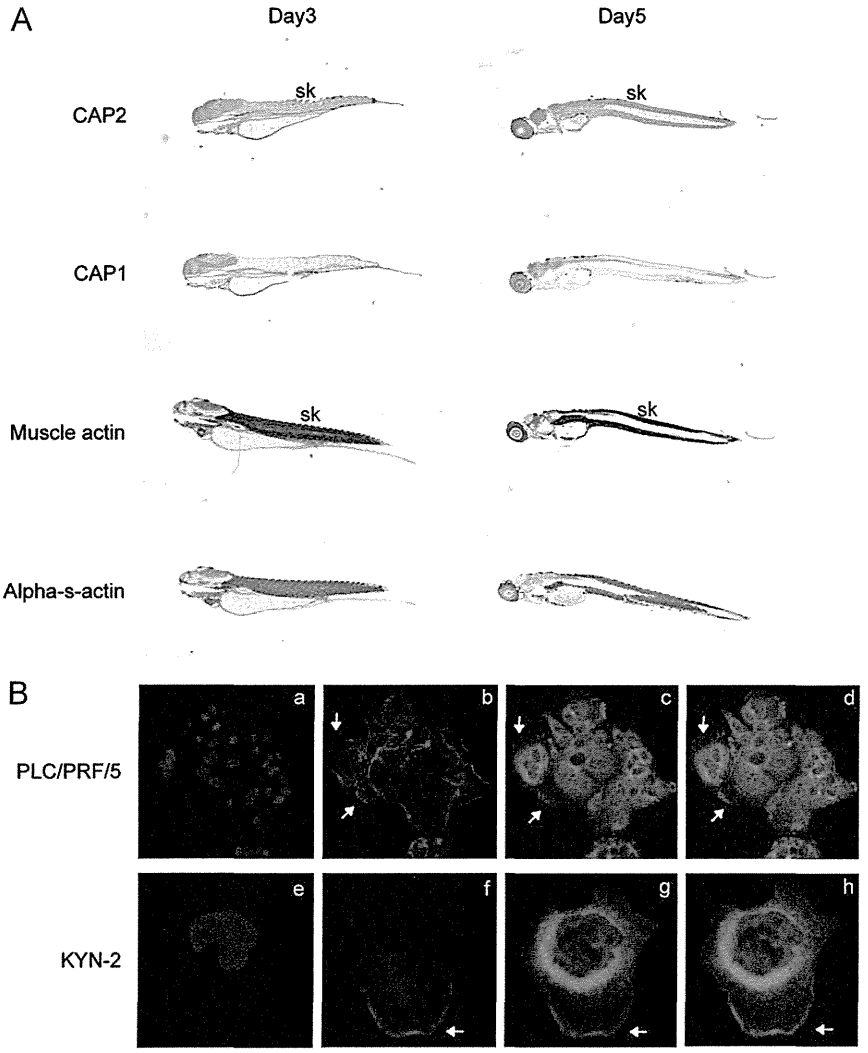

Fig. 3 – Cyclase-associated protein 2 (CAP2) observed co-localized with actin in zebrafish and hepatocellular carcinoma (HCC) cell lines. (A) Day 3 and day 5 of zebrafish larva with CAP2, CAP1 and actin immunostaining. CAP2 was highly expressed in the skeletal muscle area compared with CAP1. Immunostaining of muscle actin and alpha-sarcomeric actin was also observed in the zebrafish skeletal muscle area, suggesting co-localization of skeletal muscle actin and CAP2, magnification 20×. (B) Immunocytochemistry of PLC/PRF/5 and KYN-2 HCC cell lines. CAP2 expression was observed co-localized with the lamellipodium as a characteristic feature of motile cells, marked by white arrow (a,e, Hoechst: blue; b,f, anti-phalloidin-rhodamine: red; c,g, anti-CAP2: green; d,h, merged). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and was particularly expressed in the heart and skeletal muscle. This is in accordance with previous reports showing that CAP1 is widely expressed in nearly all organs, while CAP2 expression is localized in the brain, heart, and skeletal muscle in rat tissue [8,28]. In mouse embryonic development, the expression of CAP2 is highly restricted to the developing muscle tissues and heart [12]. A new report has shown CAP2 expression in the cardiac primordial and in the leading edge of the myotome during early *Xenopus* embryogenesis [29]. We found that knockdown of CAP2 expression in the zebrafish resulted in a shorter body compared with the control embryos. There was an abundant original expression and a co-localization of CAP2 and actin in the zebrafish skeletal muscle. CAP2 appears to act as a striated muscle-specific protein during zebrafish development. Thus, the short-body phenotype with pericardial edema observed after CAP2 knockdown in zebrafish was likely caused by the down regulation of actin-associated CAP2. This indicates that CAP2 plays an important conserved role in higher vertebrates, particularly in the development of skeletal muscle. We realized that almost all knockdowns of various genes in zebrafish affect body development [30–32], through skeletal muscle, somite, or neural formation defects, indicating that many genes or signaling pathways are involved in the patterning processes in a vertebrate embryo; here we showed the possible role of CAP2. Further, it would be interesting to investigate the mechanisms regulating CAP2 and CAP1 in developmental processes and in different organs.

Previously, using oligonucleotides array technology we identified *CAP2* as one of the genes upregulated in early HCC [2]. Overexpression of CAP2 was observed in a stepwise manner

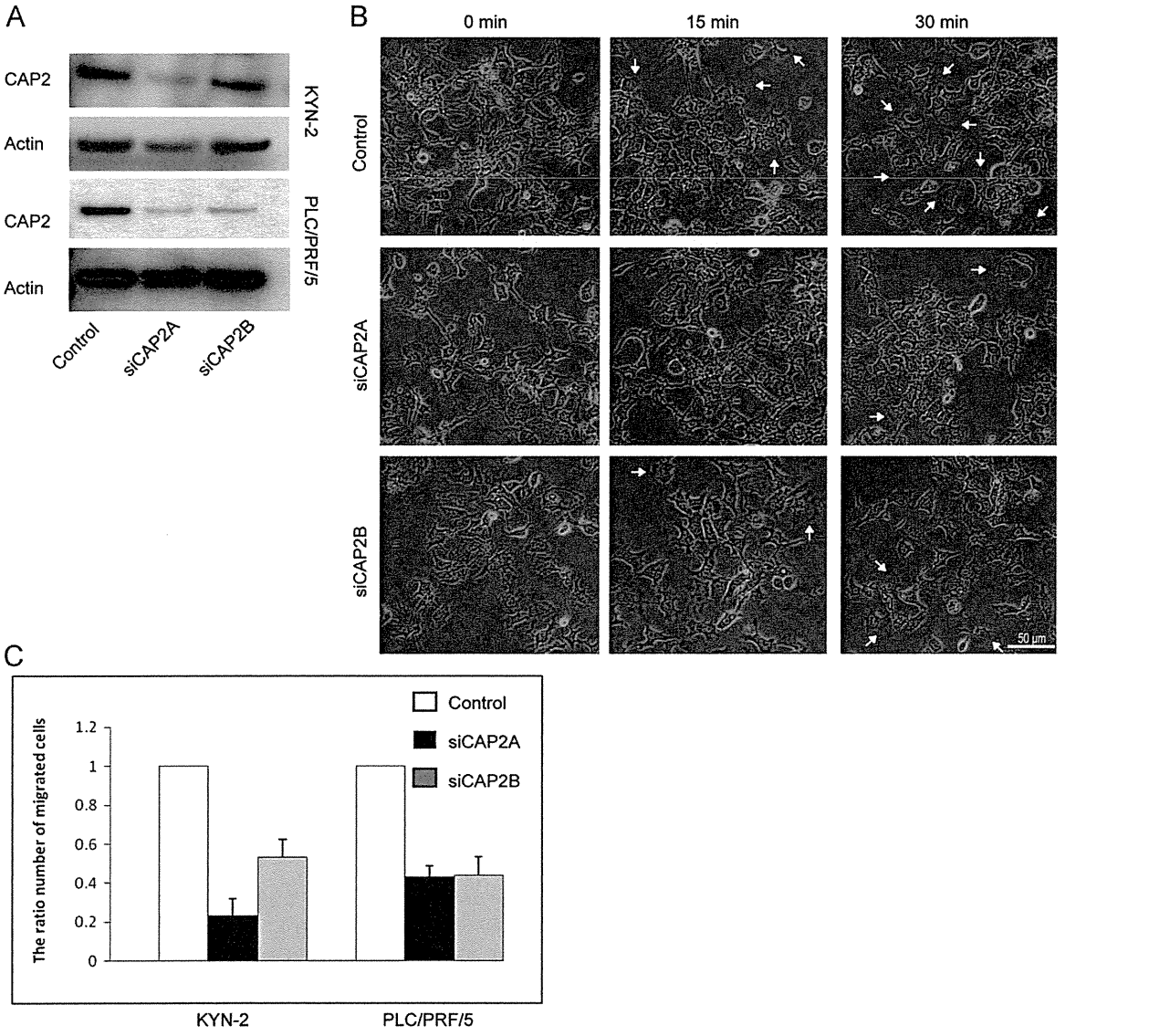

Fig. 4 – Cyclase-associated protein 2 (CAP2) silencing affected lamellipodium formation and cell migration. (A) Western blot analysis to confirm the suppression of CAP2 in KYN2 and PLC/PRF/5 cell lines. (B) Serum stimulation analysis of KYN-2 cells. After serum-starving (0 min), the lamellipodium of control cells was soon established following serum-stimulation (15 and 30 min) compared with the CAP2-knockdown cells, siCAP2A and siCAP2B. White arrows indicated the lamellipodium formation. Scale bar=50 μm. (C) Migration assay showing a reduction in the migration of both KYN-2 and PLC/PRF/5 cells, compared with the control cells. The ratio of the number of migrated cells represents the mean number (under microscope observation) of migrated cells per field against control cells. Bars reveal standard error of the mean (SEM).

during the progression of HCC and interestingly, in early HCC, the tumor cells invading the stroma stained positive for CAP2 and were clearly highlighted [14]. Here, we further investigated the co-localization of CAP2 and actin in the leading edge of the lamellipodium of HCC cells. Extension of the lamellipodium following serum stimulation was inhibited, and cell motility was reduced in CAP2 knockdown cells. This suggests CAP2 involvement in remodeling of the actin filament that occurs at the leading edge of lamellipodium as a characteristic feature of motile cells. The polymerization of actin is essential for motile processes and is regulated by some signaling pathway for actin-binding protein [33,34]. Thus, CAP2 seems to play a role in cell motility, resulting in promoting skeletal muscle development, and stromal invasion in early HCC. Moreover, we observed a significant correlation between overexpression of CAP2 and progressed features in HCC, such as tumor size, poor differentiation, portal vein invasion, and intrahepatic metastasis. This suggests that CAP2 overexpression is involved in promoting the invasive behavior of HCC cells. The role of CAP2 in other human cancers is, however, not yet clear. Human CAP2 can also interact with CAP1 and actin [35]. We have reported that the overexpression of CAP1 in pancreatic cancers is also related to the aggressive behavior of pancreatic cancer cells [36]. Further investigation of cellular and molecular mechanisms governing CAP2 role in cell migration ability is necessary to expand our knowledge on how actin dynamics is controlled. Elucidating the

Table 2 – Immunohistochemical analysis of cyclase-associated protein (CAP2) in hepatocellular carcinoma (HCC) (n=105).

Histology	CAP2 staining score		
	2+	1+	–
Well differentiated HCC (n=21)	2(10%)	9(43%)	10(48%)
Early HCC (n=9)	0(0%)	3(33%)	6(67%)
Moderately differentiated (n=68)	30(44%)	35(51%)	3(4%)
Poorly differentiated (n=16)	10(63%)	4(25%)	2(13%)

Table 3 – Correlations between clinicopathological characteristics and cyclase-assoicated protein 2 (CAP2) expression in HCC clinical cases.

Characteristics	CAP2 expression		
	–, 1+	2+	*P* value
Mean age (years)			NA[a]
Gender			0.810
Male	51	36	
Female	10	8	
Etiology			
Hepatitis B virus			0.010
Negative	51	27	
Positive	10	17	
			0.052
Negative	23	25	
Positive	38	19	
AFP serum level (ng/ml)			0.492
<20	36	23	
≧20	25	21	
Fibrosis			0.073
Liver cirrhosis	24	23	
Others	37	17	
Tumor size (cm)			0.004
<2.0	24	6	
≧2.0	37	38	
Tumor differentiation			0.001
Well (early)	19(9)	2(0)	
Moderately	37	31	
Poorly	5	11	
Portal involvement			0.001
–	37	12	
+	24	32	
Intrahepatic metastasis			0.002
–	53	27	
+	8	17	

[a] Not available.

conserved collaborative role of CAP2 and CAP1, and whether they interact with the RAS signaling pathway may also reveal their role in human cancers. Additionally, the use of zebrafish to study hepatocarcinogenesis offers a new innovative research approach [37]. A previous report has shown conserved gene expression profiles between human and zebrafish liver cancer [38]. Future studies on how CAP2 functions in zebrafish liver are necessary to advance our understanding of hepatocarcinogenesis. Combining developmental and cancer studies will provide an exciting future framework to understand how development and carcinogenesis are linked.

In summary, we showed that CAP2 has an important conserved function across species. In zebrafish, the role of CAP2 appears to be associated with the developmental process, particularly in skeletal muscle development. In HCC, CAP2 was associated with enhanced cell motility, and was involved in the progression of HCC. The role of CAP2 in the developmental process of higher vertebrates as well as in human liver cancer is shedding some light on the progress issue of development and cancer.

Conflict of interest

The authors declare there is no conflict of interest regarding this study.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2012.09.013.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Accumulation of platelets in the liver may be an important contributory factor to thrombocytopenia and liver fibrosis in chronic hepatitis C

Reiichiro Kondo · Hirohisa Yano · Osamu Nakashima · Ken Tanikawa · Yoriko Nomura · Masayoshi Kage

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Abstract
Background Thrombocytopenia is a marked feature of chronic liver disease and cirrhosis. We tried to clarify whether an accumulation of platelets in the liver contributes to thrombocytopenia and liver fibrosis in chronic liver disease.
Methods Thirty-eight patients who underwent hepatectomy for hepatocellular carcinoma (HCC) with hepatitis C virus infection were included. The locations of platelets and Kupffer cells and the expression of platelet-derived growth factor (PDGF) receptor-β and smooth muscle actin (SMA) were identified by immunohistochemistry. Perisinusoidal mesenchymal cells that express PDGF receptor-β and SMA were interpreted as transformed hepatic stellate cells (HSCs).
Results Patients with cirrhosis had a more extensive platelet area in the liver compared to controls (5601 ± 5611 vs. 564 ± 361 μm^2, $p = 0.02$), although the blood platelet count significantly decreased along with the progression of liver fibrosis. In cirrhotic liver, most platelets were present in the sinusoidal space of the periportal area with inflammation, where HSCs expressing PDGF receptor-β were frequently observed. In addition, the platelet and Kupffer cell areas were significantly smaller in cancerous tissue than those in noncancerous tissues (platelet area: 492 ± 823 vs. 3643 ± 4055 μm^2, $p = 0.001$; Kupffer cell area: 450 ± 841 vs. 3012 ± 3051 μm^2, $p = 0.001$).
Conclusions The accumulation of platelets in the liver with chronic hepatitis may be involved in thrombocytopenia and liver fibrosis through the activation of HSCs. In addition, our findings also indicate that both platelets and Kupffer cells decrease in HCC tissues.

Keywords Platelet · PDGFR-β · Hepatic stellate cells · Sinusoidal endothelial cells

Introduction

Blood platelets, besides hemostatic properties, have the features of inflammatory cells. Blood platelets, while activated in inflammatory processes, release active compounds: platelet-derived growth factors (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, and so forth [1]. Platelets transport these active compounds to the target cells [2, 3]. There are many reports presenting multipotential properties of blood platelets, such as angiogenesis [4–6], wound healing [7, 8], liver regeneration [9], and metastasis in cancer [6, 10, 11]. In acute viral hepatitis, platelets mediate cytotoxic T lymphocyte-induced liver damage [12]. After virus infection, platelets were recruited to the liver, delaying virus elimination and increasing immunopathological liver cell damage [13].

In chronic hepatitis, the blood platelet level gradually decreases, which is reflected in liver fibrosis. Blood

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platelets and chronic liver disease are also closely related. Thrombocytopenia is a marked feature of chronic liver disease and cirrhosis. Cirrhosis is a major cause of morbidity and mortality in many countries, and results in liver failure, portal hypertension, and increased risk of carcinogenesis [14]. The main causes of liver fibrosis include chronic hepatitis C virus infection, alcohol abuse, and nonalcoholic steatohepatitis [15]. The role of platelets in the pathogenesis of liver damage and the exact mechanisms leading to thrombocytopenia in cirrhosis are not yet clear [16]. The thrombocytopenia that occurs in cirrhosis is most likely due to various processes, including increased splenic platelet breakdown [17–19], splenic pooling [20, 21], and the inability of the bone marrow to increase platelet production adequately [22]. According to previous reports assessing the feasibility of platelet scintigraphy, an accumulation of platelets in the liver was observed in patients presenting with thrombocytopenia [17–20, 23–25]. Based on these findings, thrombocytopenia with chronic hepatitis and cirrhosis may be caused by hypersplenism, as well as by the capture of platelets by the liver. However, the platelet kinetics of patients with chronic liver disease are not well characterized. Therefore, the aim of the study described in the present paper was to clarify the histopathological findings of platelets in human liver tissue and to elucidate the role of platelets in the pathogenesis of chronic liver disease.

Methods

Tissues

We studied 38 patients who underwent hepatectomy for hepatocellular carcinoma (HCC) with hepatitis C viral infection at the Kurume University Hospital; their clinical backgrounds are shown in Table 1. These cases did not receive preoperative anticancer therapies. Both cancerous tissues and adjacent noncancerous liver tissues were obtained by surgical operation.

Five specimens of liver tissues obtained from patients who underwent hepatectomy for hepatic cavernous hemangioma without chronic hepatitis were used as controls.

The study was performed with informed consent obtained from patients for the use of their liver tissues in the investigation, and was approved by the ethical committee at Kurume University (approved ID number: 11200).

Histopathology

Each tissue was fixed with 10 % formalin, embedded in paraffin, cut into 5 μm sections, and then used for histological and immunohistological analyses. The specimens were stained with hematoxylin and eosin and examined under a light microscope. The histological liver damage of these specimens was evaluated for fibrosis and inflammation according to the classification proposed by the International Association for the Study of the Liver [26, 27]. The severity of fibrosis (stage of disease) was classified as none (stage 0), mild (portal fibrosis; stage 1), moderate (periportal fibrosis; stage 2), severe (bridging fibrosis with lobar distortion; stage 3), and cirrhosis (stage 4), and the inflammatory activity (grade of disease activity) was classified as none (grade 0), minimal (grade 1), mild (grade 2), moderate (grade 3), or severe (grade 4). The pathological features of HCC were evaluated according to the World Health Organization classification [28].

Histopathological diagnosis and classification were performed by four pathologists (R.K., H.Y., O.N., and M.K.).

Table 1 Clinical features of the patients studied in this work

Stage	1	2	3	4
No. of subjects	10	10	8	10
Sex (male/female)	8/2	9/1	6/2	5/5
Age (years)[a]	74 ± 5.0	71 ± 6.9	69 ± 9.9	74 ± 4.2
Grade (1/2)	7/3	3/7	1/7	1/9
Platelet count ($\times 10^4$)[a]	15.8 ± 3.4	13.5 ± 2.5	11.8 ± 5.1	10.6 ± 2.5
HCC (well/moderate/poor)	0/10/0	1/8/1	1/7/0	0/10/0

The severity of fibrosis was classified as none: stage 0, portal fibrosis: stage 1, periportal fibrosis: stage 2, bridging fibrosis with lobar distortion: stage 3, and cirrhosis: stage 4. The inflammatory activity was classified as none: grade 0, minimal: grade 1, mild: grade 2, moderate: grade 3, or severe: grade 4

HCC hepatocellular carcinoma, *well* well differentiated HCC, *moderate* moderately differentiated HCC, *poor* poorly differentiated HCC

[a] Mean ± SD

Immunohistochemical analysis

The avidin–biotin peroxidase complex method was used for immunohistochemistry. We used monoclonal antibodies against CD41 (1:500, Beckman Coulter, Brea, CA, USA), CD68 (1:200, DAKO, Glostrup, Denmark), PDGF receptor-β (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and smooth muscle actin (1:200, DAKO). CD41 (glycoprotein IIb/IIa complex) is a specific marker for platelets, so a CD41-positive reaction was taken to indicate the presence of platelets. CD68, an anti-human macrophage antibody, is expressed not only in residential macrophages such as Kupffer cells, but also in migrating macrophages. Among

the CD68-positive cells, those in the sinusoidal space or blood space of cancerous tissues with spindle or stellate-shaped cytoplasm, and those partly adhering to the sinusoidal endothelial cells, were evaluated as Kupffer cells. Perisinusoidal mesenchymal cells express PDGF receptor-β as transformed hepatic stellate cells (HSCs) [29–31]. These cells were evaluated as activated HSCs.

Measurement of cells

The area of platelets and Kupffer cells in each specimen was measured using the WinROOF software package (version 6.1, Mitani Corporation, Fukui, Japan). In noncancerous liver tissues, the areas were measured in five randomly selected periportal regions. In cancerous tissues, five visual fields were randomly selected.

Transmission electron microscopy

The liver was cut into small pieces (approximately 1 mm^3), the specimens were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and they were then post-fixed in 1 % O_8O_4 in 0.1 M phosphate buffer. Next, the specimens were dehydrated through a graded series of ethanol, passed through propylene oxide, and embedded in Epok 812. Ultrathin sections mounted on copper grids were stained with uranyl acetate and lead citrate and observed in a Hitachi (Tokyo, Japan) H-7650 transmission electron microscope.

Statistical analysis

The arithmetic means and standard deviations of our data were calculated using the JMP software package (release 9.0, SAS Institute, Cary, NC, USA). All data are expressed as mean $\pm$ SD, and p values of less than 5 % were considered to indicate significance.

Results

Histological findings

Noncancerous liver tissues of all patients with hepatitis C virus infection showed various degrees of fibrosis and chronic inflammation. The severity of fibrosis was mild (stage 1) in ten patients, moderate (stage 2) in ten patients, and severe (stage 3) in eight patients, while ten patients had cirrhosis (stage 4). The inflammatory activity was minimal (grade 1) in 12 patients and mild (grade 2) in 26 patients. In control tissues, there were a few lymphocytes, but only in the portal area, and neither necroinflammatory reactions nor fibrosis were noted (grade 0, stage 0).

Among the cancerous tissues in 38 cases, 35 cases were moderately differentiated HCCs, two cases were well-differentiated HCCs, and one case was poorly differentiated HCC. In terms of the diameters of the HCCs, there were two cases with an HCC diameter of $\leq$1.0 cm, 11 cases with a diameter of 1.1–2.0 cm, 14 cases with 2.1–3.0 cm, and 11 cases with $\geq$3.0 cm. Comparing the histological differentiation of cancerous tissues, the mean tumor size was 2.6 $\pm$ 0.6 cm in the well-differentiated HCCs, 3.0 $\pm$ 1.8 cm in the moderately differentiated HCCs, and 6.3 cm in the poorly differentiated HCCs. Among the 37 nodular-type HCCs, 24 specimens had clear fibrous capsules at the tumor and nontumor boundary, while 13 cases had no fibrous capsules.

Platelets in noncancerous liver tissues

In all noncancerous liver tissues, including patients with chronic hepatitis or cirrhosis and in the controls, there were platelets but no megakaryocytes in the sinusoidal space. Patients with cirrhosis had a more extensive platelet area in the noncancerous liver tissue than in controls (5601 $\pm$ 5611 vs. 564 $\pm$ 361 μm^2, $p = 0.02$, $p < 0.05$, Fig. 1a). In patients with chronic hepatitis or cirrhosis, the platelet area in noncancerous liver tissues increased along with an increase in histological liver damage ($p = 0.02$, $p < 0.05$), although the blood platelet count significantly decreased (Fig. 1b). In noncancerous liver tissues with chronic hepatitis or cirrhosis, platelets were present predominantly in the sinusoidal space of the periportal area with inflammation. In high-stage cases, platelets were observed along the destroyed limiting plate of the expanded fibrous portal area with inflammation, and in the sinusoidal space of the periportal area (Fig. 2).

Relationship among platelets, HSCs, and Kupffer cells in non-cancerous liver tissues

Immunohistochemical studies of noncancerous liver tissues with cirrhosis revealed that most platelets were present in the periportal area with inflammation, where HSCs expressing PDGF receptor-β were frequently observed (Fig. 3a, b). Most smooth muscle actin stained cells were identical to those expressing PDGF receptor-β (Fig. 3c). In noncancerous liver tissues of controls and cases at the lower stage of chronic hepatitis, only a few HSCs expressing PDGF receptor-β were seen.

In noncancerous liver tissues, including patients with chronic hepatitis or cirrhosis and in controls, CD68-positive Kupffer cells were seen in the sinusoidal spaces of both periportal and lobular areas diffusely.

Platelets and Kupffer cells in cancerous tissues

In all cancerous tissues, a few platelets and Kupffer cells were present in the blood spaces of cancerous tissues.

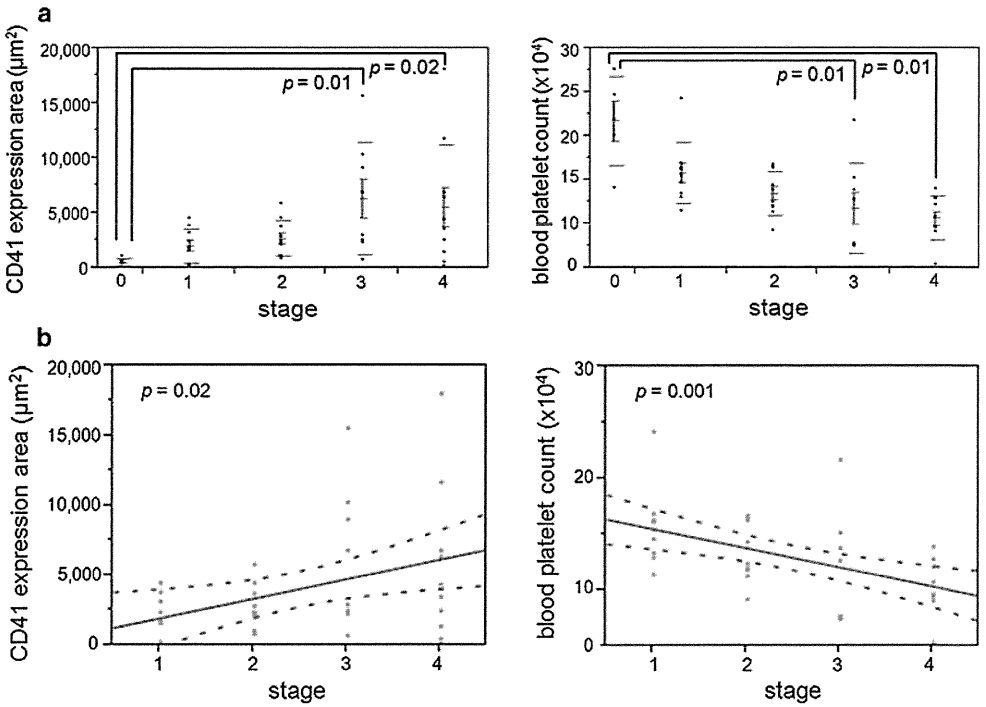

Fig. 1 Relationship between histological liver damage and either the CD41 expression area in the noncancerous liver tissues or the blood platelet count. **a** The noncancerous liver tissues with cirrhosis had a larger CD41 expression area than seen in the controls ($p = 0.02$, $p < 0.05$), although the blood platelet count was small. **b** In the noncancerous liver tissues with chronic hepatitis and cirrhosis, the CD41 expression area increased with increasing histological liver damage ($p = 0.02$, $p < 0.05$), although the blood platelet count decreased ($p = 0.001$, $p < 0.05$)

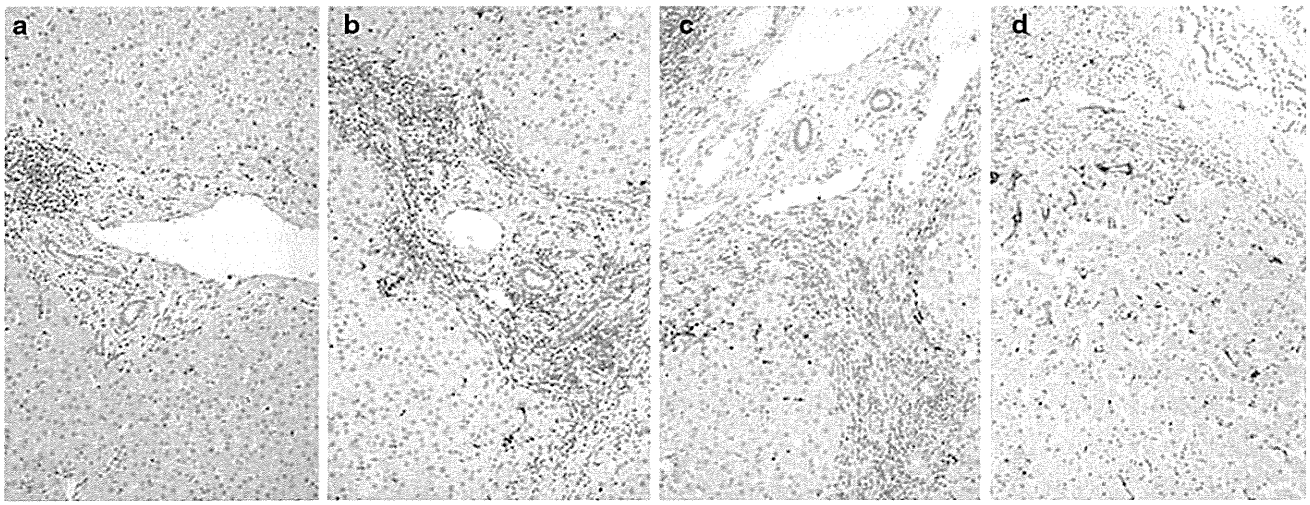

Fig. 2 Typical immunostaining for CD41 in a periportal area of noncancerous liver tissues with chronic hepatitis or cirrhosis. In noncancerous liver tissues with chronic hepatitis or cirrhosis, CD41-positive reactions are present predominantly in the sinusoidal space of the periportal area with inflammation. In high-stage cases, strong CD41 positive reactions are observed along the destroyed limiting plate of the expanded fibrous portal area with inflammation, and in the sinusoidal space of the periportal area. Labeled streptavidin biotin with CD41 antibody: **a** grade 1, stage 1; **b** grade 2, stage 2; **c** grade 2, stage 3; **d** grade 2, stage 4

When the platelet and Kupffer cell areas in the cancerous and non-cancerous tissues were compared, the platelet and Kupffer cell areas were significantly smaller in cancerous tissue than in noncancerous tissue (Fig. 4a, platelet area: 492 ± 823 vs. 3643 ± 4055 μm^2, $p = 0.001$, $p < 0.05$; Fig. 4b, Kupffer cell area: 450 ± 841 vs. 3012 ± 3051 μm^2, $p = 0.001$, $p < 0.05$). Regardless of the liver damage in noncancerous tissue, both the platelet and Kupffer cell areas in cancerous tissue were smaller than those in noncancerous tissue. The platelet and Kupffer cell areas in cancerous tissue tended to decrease with decreasing histological differentiation of HCC (Table 2). We also measured with regard to the

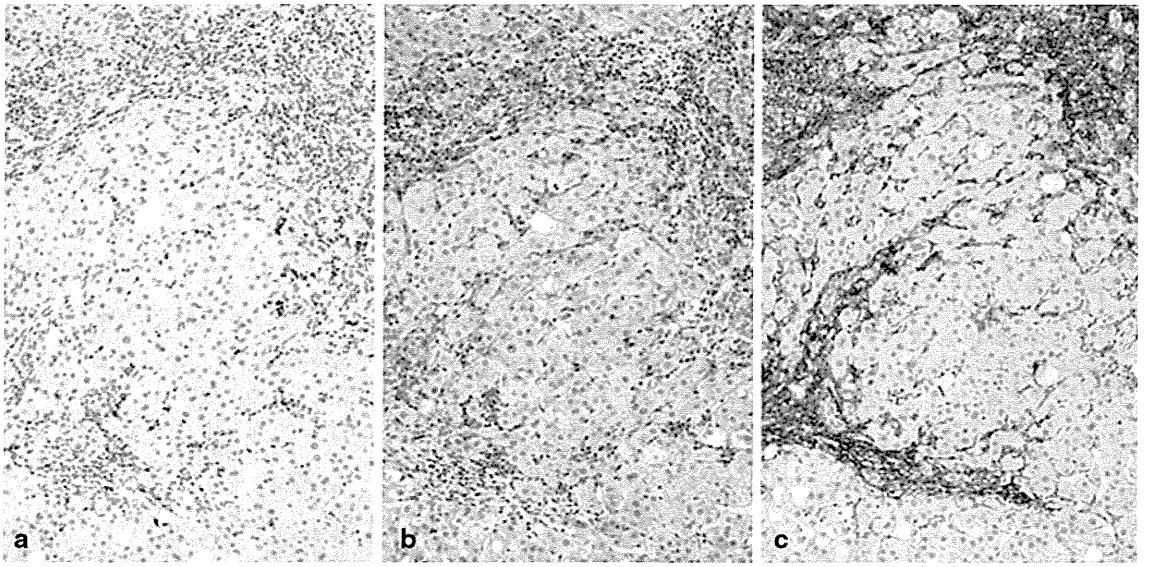

Fig. 3 Typical immunostaining for CD41, PDGF receptor-β, and smooth muscle actin in the periportal area of noncancerous liver tissues with cirrhosis. **a** Platelets are present in the periportal area with inflammation. Labeled streptavidin biotin with CD41 antibody. **b** Serial section shown in **a** stained for PDGF receptor-β antibody. Portal mesenchymal and perisinusoidal cells are stained for PDGF receptor-β. These are dense in the periportal area, where platelets are frequently observed in **a**. Labeled streptavidin biotin with PDGF receptor-β antibody. **c** Serial section shown in **b** stained with smooth muscle actin. Most of the stained cells are identical to those stained for PDGF receptor-β in **b**. Labeled streptavidin biotin with smooth muscle actin antibody

Kupffer cell count. The results evaluated by Kupffer cell count correlate with those evaluated by Kupffer cell areas. The Kupffer cell count was significantly smaller in cancerous tissue than in noncancerous tissue (data was not shown).

Electron microscopic findings

In the sinusoidal space of noncancerous tissues with cirrhosis, there were platelets with dense granules and α-granules. They were partly attached to the sinusoidal endothelial cells (Fig. 5a). Some of the platelets had several empty granules, which are indicative of degranulation (Fig. 5b). Platelets were rarely found in the space of Disse. Around platelets that adhered to the sinusoidal endothelial cells, HSCs that were more spindle-shaped and had a few fat droplets were observed (Fig. 5c). Platelets were not in direct contact with HSCs and Kupffer cells.

Discussion

Blood platelets, by connecting hemostasis and inflammatory processes, participate in the pathogenesis of chronic liver disease. In this study, we demonstrated the pathological findings for the accumulation of platelets in the liver in cases with chronic hepatitis C.

Hill-Zobel et al. [32] studied platelet dynamics in healthy humans using ^{111}In-labeled platelets. They reported

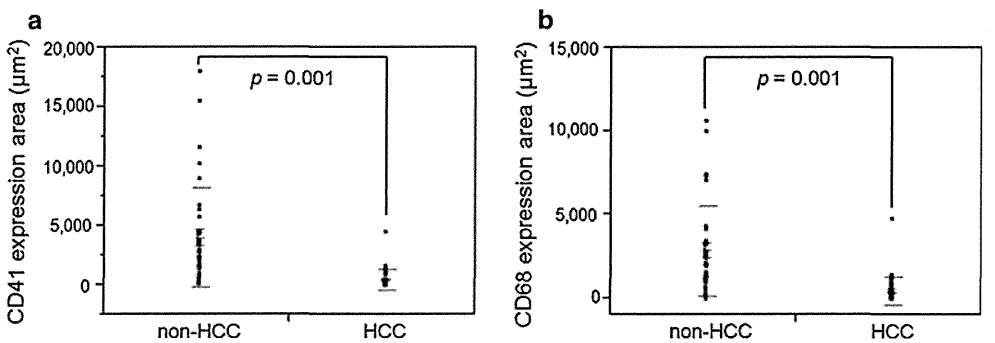

Fig. 4 CD41 (**a**) and CD68 (**b**) expression areas in different tissue types (HCC vs. non-HCC). Both the platelet and Kupffer cell areas are significantly smaller in HCC tissue than those in nonHCC tissue (platelet area: 492 ± 823 vs. 3643 ± 4055 μm^2, $p = 0.001$, $p < 0.05$; Kupffer cell area: 450 ± 841 vs. 3012 ± 3051 μm^2, $p = 0.001$, $p < 0.05$)

that the uptake in the liver was 12.6 % at 30 min and 24 % at ten days, and that in the spleen was 42.7 % at 30 min and 37 % at ten days in healthy humans [32]. The accumulation of platelets in the normal control liver tissue was confirmed in this study. However, the platelet dynamics of patients with chronic hepatitis or cirrhosis are not yet clear. In the current study, we found that the accumulation of platelets in noncancerous liver tissues of patients with chronic hepatitis or cirrhosis increased with increasing histological liver damage. In patients with chronic hepatitis or cirrhosis, the blood platelet level gradually decreased, and was reflected in the liver damage. As there are no megakaryocytes in liver tissues, we can distinguish between platelets that have accumulated in the sinusoidal space and are derived from bone marrow from those derived from extramedullary hematopoiesis. The extramedullary hematopoietic tissue should have hematinic cells, such as megakaryocytes, and immature cells. The accumulation of platelets in the cirrhotic liver with thrombocytopenia has been shown using platelet scintigraphy [17, 23–25]. In patients with thrombocytopenia, Kinuya et al. [24] reported that the spleen/liver uptake ratio for ^{111}In- or ^{99}Tc-labeled platelets was apparently lower in patients for whom splenectomy is ineffective than in those for whom it was effective. Sata et al. [25] reported that platelets were captured in the liver during interferon therapy for chronic hepatitis B, and that this phenomenon is one cause of the decrease in peripheral blood platelets during interferon therapy. The results of our present study are not necessarily contrary to the established theory of thrombocytopenia with chronic hepatitis. It has been reported that the mechanisms leading to thrombocytopenia in cirrhosis most likely involve multifactorial processes [17–22]. We consider that the accumulation of platelets in the liver with chronic hepatitis and cirrhosis may be one of the important contributory factors to thrombocytopenia.

Table 2 Relationship between histological differentiation and platelet area or Kupffer cell area in HCCs

Histological differentiation	Platelet area (μm^2)	Kupffer cell area (μm^2)
Well ($n = 2$)[a]	1532 ± 26	2674 ± 3094
Moderate ($n = 35$)[a]	440 ± 82	305 ± 405
Poor ($n = 1$)	225	108

well well differentiated HCC, *moderate* moderately differentiated HCC, *poor* poorly differentiated HCC

[a] Mean ± SD

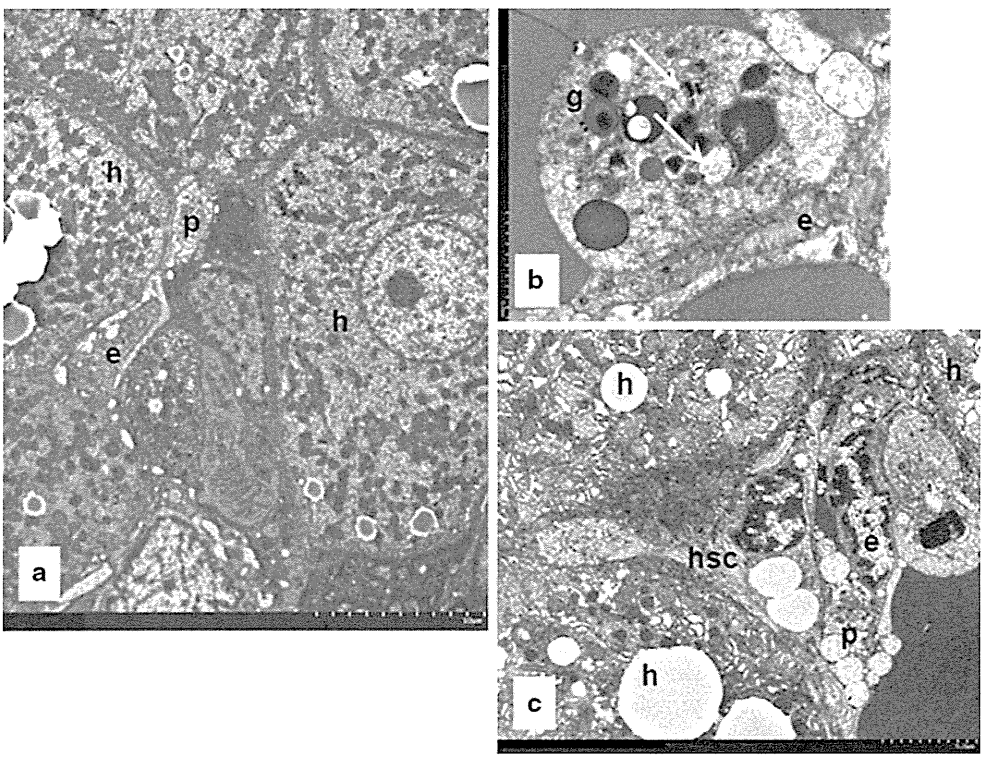

Fig. 5 Electron micrographs of a cirrhotic liver. **a** Platelets (*p*) in the sinusoidal space adhere to endothelial cells (*e*). **b** A platelet that has adhered to a sinusoidal endothelial cell contains both α-granules (*g*) and empty ones (*arrows*). **c** Around platelets that have adhered to a sinusoidal endothelial cell, a hepatic stellate cell (*hsc*)—which is more spindle-shaped, and has a few fat droplets—can be observed. *h* hepatocytes; **a** ×5000, **b** ×25000; **c** ×7000

Histologically, platelets in noncancerous liver tissues of patients with chronic hepatitis or cirrhosis are seen predominantly in the sinusoidal space of the periportal area with inflammation. As viewed through an electron microscope, the platelets aggregate in the sinusoids and adhere to the sinusoidal endothelial cells. Platelets contain four types of distinguishable granules or vesicles: dense granules, lysosomes, peroxisomes, and α-granules. These granules contain various active compounds. Blood platelets, activated in inflammatory and immune processes, release different intraplatelet compounds [1]. Platelets transport these active compounds in their granules to the target cells [2, 3]. Platelets then adhere to endothelial cells or exposed subendothelial matrices. Following adhesion, they become activated and secrete granule content. The accumulation of platelets in the liver and the adhesion to the sinusoidal endothelial cells are important steps in direct platelet action for the liver. In blood vessels, the vessel wall, with its inner lining of endothelium, is crucial to the maintenance of a patent vasculature. The endothelium contains thromboregulators—nitric oxide, prostacyclin, and the ectonucleotidase CD39—which together provide a defence against platelet thrombus formation [33]. When the endothelium is disrupted, collagen and tissue factor become exposed to the flowing blood, thereby initiating the formation of thrombus. Endothelium is also an important target for tumor necrosis factor (TNF) and interleukin-1 (IL-1). The endothelium synthesizes and releases platelet activating factor (PAF) in response to TNF. This activity of TNF overlaps that of IL-1, which also induces PAF production in endothelium [34]. These vessel wall alterations result in a change in endothelium from antithrombotic to thrombotic. The disrupted endothelium is the first reaction in platelet adhesion to the vessel subendothelium under physiologic blood flow [33]. In the presence of TNF-α-induced sinusoidal alteration, platelets adhere to sinusoidal endothelial cells in the same way as to vessel walls [35]. Characteristic pathological features of chronic HCV infection are chronic inflammation and apoptosis of infected and bystander hepatocytes [36, 37]. In a model of hepatitis, Kupffer cells produced the majority of TNF-α [38]. Under lipopolysaccharide administration in mice, TNF or IL-1 induce platelets to accumulate in the liver sinusoidal space within a few minutes by a different mechanism of aggregation [39–41]. With the depletion of Kupffer cells, platelets do not accumulate in the liver after lipopolysaccharide administration [41]. Kupffer cells are involved in various mechanisms, such as phagocytosis, metabolism, cytokine generation, and antitumor effect. Platelets may accumulate in the sinusoidal space under thrombocytotic conditions in chronic hepatitis C brought about by the activated hepatic reticuloendothelial system, as caused by inflammation through the mechanism involving the activation of Kupffer cells.

The role of platelets in the pathogenesis of chronic hepatitis and cirrhosis is not clear. We suggest that platelet-derived factor and liver condition should be taken into account when examining the role of platelets in the liver. We identified a dense population of cells expressing PDGF receptor-β in the periportal areas of cirrhotic liver, whereas only a few mesenchymal cells stained for this peptide were seen in patients at the lower stage of chronic hepatitis and in controls. In addition, most of the PDGF receptor-β expressing cells were also stained for smooth muscle actin. These cells, which play a central role in liver fibrosis, are believed to be transformed from HSCs [29–31], and their proliferation is stimulated by PDGF [42]. HSCs are increasingly being seen as key mediators in the progression of liver fibrosis. In this study, HSCs expressing PDGF receptor-β were located in the periportal area, where platelets were frequently observed. PDGF is the basic mediator involved in platelet granules. PDGF overexpression has been linked to different types of fibrotic disorders and malignancies [43]. In chronic liver disease, the essential role of all PDGF family members in liver fibrosis has been demonstrated [42, 44–47]. To date, four members of the PDGF family have been identified: PDGF-A, PDGF-B, PDGF-C, and PDGF-D [48]. PDGF-B has a stimulating influence on the fibrogenesis and mitogenesis of HSCs in the liver [42]. The biological effects of PDGF are elicited through binding to two specific receptors, PDGF receptor-α and PDGF receptor-β. The binding affinity of PDGF receptor-β is restricted to PDGF-B [49]. When HSCs expressing PDGF receptor-β are present in the liver, the liver may be susceptible to PDGF contained in platelets. The accumulation of platelets in the liver with chronic hepatitis may be involved in liver fibrosis through the activated HSCs.

We also found that the platelet area was significantly smaller in cancerous tissue than that in noncancerous tissues. It has been reported that the number of Kupffer cells in cancerous tissues decreased in comparison with the number in noncancerous tissues as the histological differentiation of HCC decreased [50]. Indeed, the Kupffer cell area in cancerous tissues was significantly smaller than that in noncancerous tissues and tended to decrease with decreasing histological differentiation of HCC in this study. We consider that platelets may also accumulate in the blood space in cancerous tissue through some mechanisms involving the Kupffer cells.

In conclusion, the results obtained in the present study indicate that the accumulation of platelets in the liver with chronic hepatitis C may be involved in thrombocytopenia and liver fibrosis through the activated HSCs. In addition, platelets may accumulate in the sinusoidal space through another mechanism involving the activation of Kupffer cells. Further study of the biological characteristics and

function of these cells will contribute to improving the treatment of thrombocytopenia and liver fibrosis.

Conflict of interest The authors declare that they have no conflict of interest.

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