Overexpression of κ-Actin Alters Growth Properties of Hepatoma Cells and Predicts Poor Postoperative Prognosis

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Abstract. *Since Rho-mediated signaling can regulate liver cancer cell proliferation, amino acid sequence changes of its downstream targets, actins, might alter the properties of cell growth. Here, we investigated the function of a novel class of actins, named κ-actins, in hepatocellular carcinoma (HCC). Materials and Methods: Alexander cells overexpressing an HCC-derived κ-actin (Alex-κ cells) were established to study growth property changes. κ-actin expression was also determined in tumor and noncancerous tissues from 72 HCC patients. Survival analysis was conducted to evaluate the prognostic predictive value of κ-actin expression. Results: Phylogenetic analysis showed that κ-actin sequences constituted 94.7% and 17.6% of actin transcripts in Alex-κ and naive Alexander cells, respectively. Alex-κ cells exhibited serum-independent cell growth with increased anchorage-independent colony formation and BrdU incorporation upon serum deprivation. Cox proportional hazard analysis showed that κ-actin expression in both cancerous and noncancerous tissues predicted poorer postoperative disease-free survival (p=0.004). Conclusion: Overexpression of κ-actin altered growth properties of hepatoma cells, contributing to poor postoperative prognosis.*

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Hepatocellular carcinoma (HCC) is the fifth most common solid cancer worldwide and the third most common cause of cancer-related death (1). The major etiologies of HCC include chronic hepatitis B virus (HBV) infection, chronic hepatitis C virus (HCV) infection, and alcoholic liver disease (2-5). Other risk factors include old age, male gender, diabetes, underlying chronic liver diseases, and most importantly, liver cirrhosis (3, 6). Because of the multifactorial etiology and heterogeneous nature of the disease, the key molecular pathways leading to hepatocarcinogenesis remain unclear. With the help of advanced technologies in genomic medicine, hepatocarcinogenesis was discovered to involve not only multiple molecular events but also heterogeneous cellular pathways (7, 8).

An important property of cancer cells is their ability to migrate and invade other tissues. This process is closely associated with the dynamic morphological changes of cytoskeletal and adhesive structures (9-12). Rho kinase (ROCK), a target of Rho, phosphorylates and activates LIM-kinase (LIMK) to inhibit cofilin-induced depolymerization of actin microfilaments. ROCK also inactivates myosin light chain (MLC) phosphatase and phosphorylates MLC to increase contractility of actin-myosin (13). As a result, the Rho/ROCK pathway increases contractile tension forming stress fibers and mature focal contacts. Recent studies have indicated that Rho-associated protein kinases play a critical role in the metastasis and tumor growth of HCC (14-18). Since Rho signaling is important in modulating liver cancer cell motility, it is conceivable that alteration of its target proteins, the actin molecules, can modify cell motility. Altered actin isoform expression occurs in some pathological conditions, including muscular malignancies. Previously, we discovered a significant increase of the proportion of non-β-

actins in HCCs (19). Among the non-β-actins, a novel group of actin mutants was identified, temporarily named κ-actins. Two genetically closely related sequences, *FKSG30* and an actin-like sequence in the Cat Eye Syndrome region, were found to cluster with the κ-actin sequences. Subsequently, it was discovered that the κ-actin sequence appeared as a hybrid peptide in a member of a primate-specific gene family, POTE, which was expressed in many types of cancer (20-24). In this study, we examined the role of κ-actin in HCC cell growth.

Materials and Methods

Cell culture. Four HCC cell lines (Huh7, HepG2, Hep3B, and Alexander (PLC/PRF/5)) were analyzed in this study. Huh7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. HepG2, Hep3B and Alexander cells were maintained in minimum essential medium containing 10% fetal bovine serum. A κ-actin sequence previously isolated from the cancerous tissue of a HCC patient encoded a protein with amino acid sequence completely identical to that of FSKG30. This κ-actin sequence was inserted into a pRc/CMV vector (Invitrogen, San Diego, CA, USA) to generate pCMV-Kappa. Stable transformants expressing κ-actin were established by transfection of pCMV-Kappa into Alexander cells.

RNA extraction, RT-PCR, and sequence analysis. RNA extraction, RT-PCR, sequence analysis and phylogenetic analysis were performed according to our previous publication (19). The sequences of the PCR products homologous to the actin gene fragment (nt. 321-630) were subjected to phylogenetic analysis. To establish phylogenetic trees, the corresponding regions of the following reference sequences (with GenBank accession numbers) were included: two α-actin mRNA sequences (J05192 and XM_001869), two β-actin mRNA sequences (NM_001101 and BC004251), two γ-actin mRNA sequences (BC001920 and BC000292), FKSG30 mRNA sequence (AY014272), an actin-like sequence located in the Cat Eye Syndrome region (chromosome 22q11.2, BA00006), and two POTE–actin fusion protein coding sequences (NM_001083538 and NM_001099771).

To detect κ-actin mRNA in culture cells or cancerous and non-cancerous tissues, primers specific to the κ-actin sequences were designed. This method was described previously with minor modification (19). For reverse transcription, the pKB-RT primer 5'-TGCCTCTCTTGCTCTGGGC-3' (nt. 274-256; antisense) was used. The sequence of this primer was completely matched to the sequences of β-actin, FKSG30 and the two POTE–actin fusion proteins mRNAs. The primers for specific amplification of κ-actin cDNA were pKapa-1, 5'-TACCGCTGTGCTCGTCATT-3' (nt. 96-111; sense), and pKapa-2, 5'-CTGATGCATGCCCCCCATCAT-3' (nt. 231-211; antisense). Among 5 POTE–actin fusion mRNA sequences (NM_001083538, NM_001099771, XM_929706, XM_002342259, XM_928585) and FKSG30 mRNA sequence, pKapa-1 was completely matched to three of them with only one nucleotide mismatch in the other three sequences. On the other hand, pKapa-2 was completely matched to four of them with only one nucleotide mismatch in the other two sequences. In contrast, when compared with β-actin mRNA sequence, five mismatches were present in pKapa-1 and four mismatches in pKapa-2.

Characterization of cell growth properties. Cell proliferation was assessed by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Culture cells were grown in a 96-well plate at a density of 5×10^3 cells per well. On the day of the assay, cells were incubated at 37˚C for 4 h in culture medium containing 0.5 mg/ml of MTT, and then lysed by dimethyl sulfoxide (DMSO). The absorbance was measured with a spectrophotometer at 570 nm.

To perform 5-bromo-2'-deoxy-uridine (BrdU) incorporation assay, cells were plated at a density of 1×10^4 per well in a 96-well plate in serum-free medium for 48 h to synchronize the cells in the G_0 phase. To start the cell cycle, 10% fetal bovine serum was added to the medium. Cells were then pulsed with 10 μM BrdU for 1 h at 3-h intervals from 0 to 27 h. The amount of incorporated BrdU at each time point was measured by use of a chemiluminescent immunoassay, the BrdU Cell Proliferation ELISA (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol. All assays were carried out in triplicate.

Cell invasion assays were performed using the Cell Invasion Assay Kit ECM 550 (Chemicon International Inc., Billerica, MA, USA) according to the manufacturer's protocol. The number of the invasive cells in 10 fields (×200) was counted under microscope and averaged. Apoptotic hepatocytes were demonstrated by the DeadEnd Fluorometric TUNEL System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

Clinicopathological analysis. Under approval of the Institutional Review Board, Chang Gung Medical Center, 72 HCC patients undergoing total resection of liver tumors from July 1997 to December 2001 in Chang Gung Medical Center, Taiwan, were included. All samples were frozen to –70˚C immediately at the Institutional Tissue Bank after surgical resection. Clinicopathological data were retrospectively reviewed, which included sex; age; HBV surface antigen (HBsAg); antibody against HCV (anti-HCV); liver cirrhosis status; alcoholism; Edmonson's histological grading; microvascular invasion; tumor capsular invasion; number of tumors; largest tumor size; macrovascular invasion; presence of ascites; prothrombin time; serum concentrations of alpha-fetoprotein (AFP), albumin, bilirubin, creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT); the date of surgical resection; the date of tumor recurrence or metastasis; and the date of last follow-up or HCC-related death.

Preoperative diagnosis of HCC was made by echo-guided liver biopsy, fine-needle aspiration cytology, or high AFP level (>200 ng/ml) plus at least two dynamic imaging studies (dynamic computed tomography, magnetic resonance imaging, or angiography). Tumors were totally removed with a safety-margin of 1 cm. HBsAg was measured by a radioimmunoassay (Ausria-II, HBsAg-RIA; Abbott Laboratories, North Chicago, IL, USA). Anti-HCV was measured by a third-generation enzyme immunoassay (HCV EIA III; Abbott Laboratories).

Results

Phylogenetic analysis of actin sequences isolated from HCC cell lines. A fragment of actin mRNA sequence was isolated from four HCC cell lines (Huh7, HepG2, Hep3B, and Alexander) by RT-PCR. After cloning into pCR2.1-TOPO vector, 20 clones with inserts were sequenced. Nucleotide

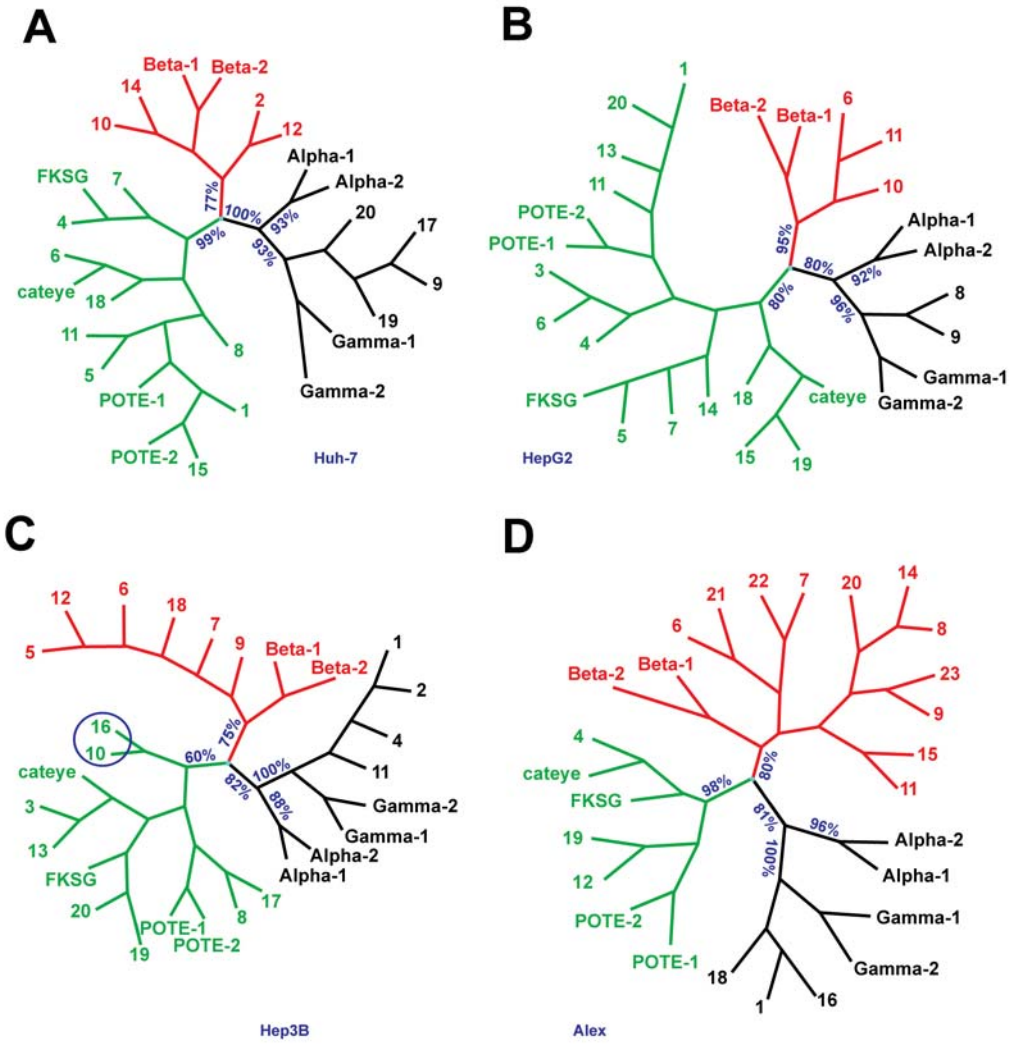

Figure 1. *Phylogenetic analysis of actin cDNA sequences derived from Huh7 (A), HepG2 (B), Hep3B (C), and Alexander (D) cells. Bootstrap analysis was performed to obtain the bootstrap value for robust separation of the main branches. Red, black, and green branches are used to represent β-actin, α- and γ-actin, and κ-actin sequences, respectively. Pseudogene sequences in (C) are circled.*

sequences homologous to actin genes were submitted for phylogenetic analysis (Figure 1; GenBank accession numbers, HM358926 to 358995). Bootstrap analysis revealed segregation of four robust groups of actin mRNA sequences with high bootstrap values. Sequences of the putative actin retroposon in POTE-actin fusion transcripts (POTE-1 and 2), FKSG30, and the corresponding gene region in Cat Eye Syndrome constituted a group separable from α-, β-, and γ-actin sequences, named κ-actin. Two sequences isolated from Hep3B cells (no. 10 and 16) completely matched a pseudogene. Otherwise, κ-actin sequences constituted 8/16 (50.0%), 13/18 (72.2%), 6/16 (37.5%), and 3/17 (17.6%) of actin transcripts in Huh7, HepG2, Hep3B, and Alexander (Alex) cells.

Alterations of cell growth in Alexander cells expressing κ-actin. Alexander cells overexpressing an HCC-derived κ-actin were established (Alex-κ cells). Sequence analysis of the actin transcripts indicated that κ-actin transcripts constituted 18/19 (94.7%) of the total actin transcripts in Alex-κ cells, compared to only 17.6% in the naive Alex cells. Both Alex cells and Alex-κ cells were submitted for cell-based assays. MTT assays indicated that the growth of Alex-κ cells was significantly reduced when compared to Alex cells (Figure 2A). No significant difference was observed in cell invasion assays (Figure 2B). Soft agar anchorage-independent growth assays showed significantly reduced colony formation in Alex-κ cells (Figure 2C). However, TUNEL assays showed less apoptosis in Alex-κ cells (Figure 2D). These results indicated that

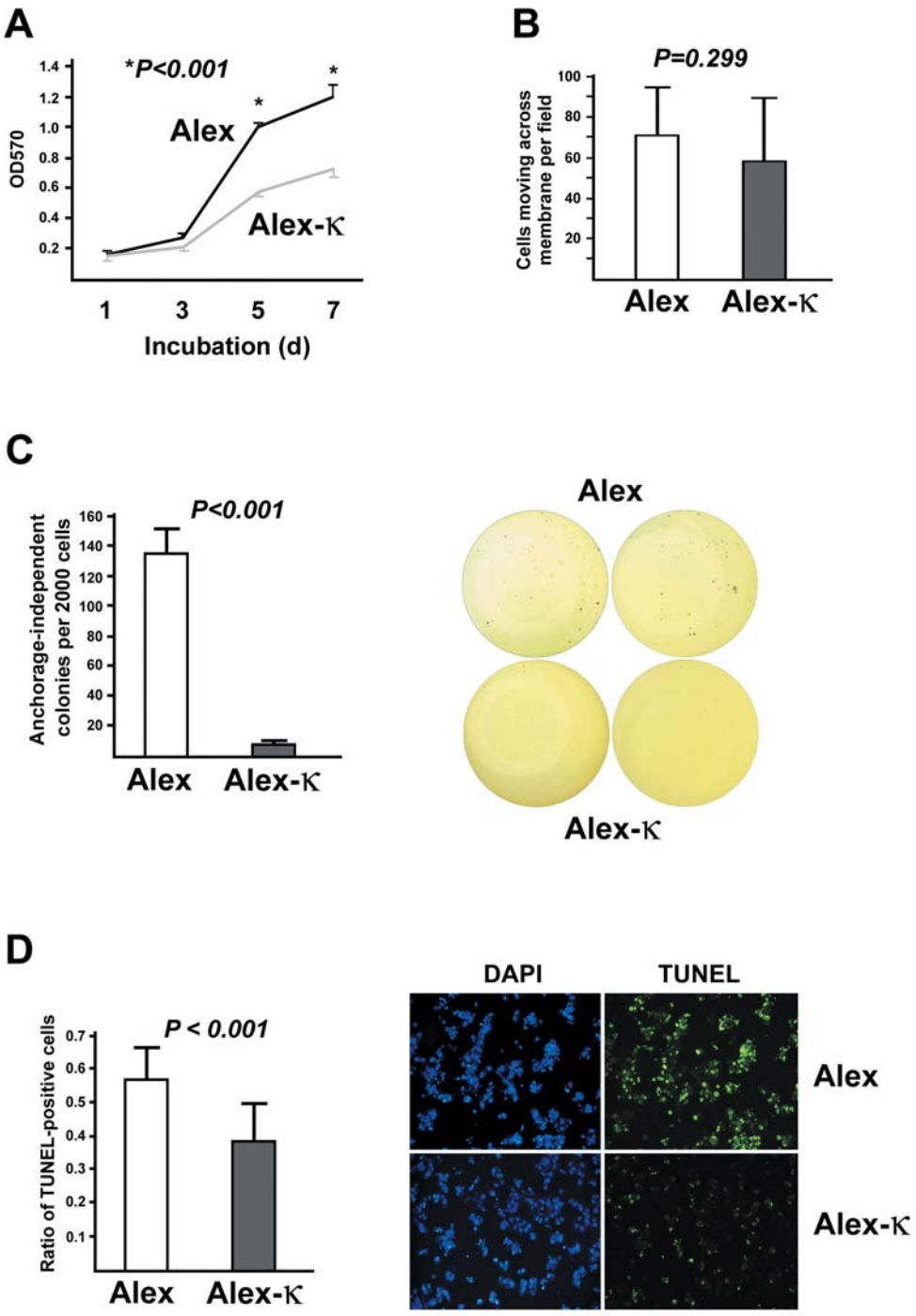

Figure 2. *Comparison of the cell growth between Alex and Alex-κ cells in the presence of 10% fetal bovine serum. A: MTT assays. B: Invasion assays. C: Efficiency of colony formation in soft agar. D: TUNEL assay for cell apoptosis.*

although Alex-κ cells grew slower and had lower invasive ability, the cells were also more resistant to apoptosis.

Subsequently, serum starvation was performed, in order to attempt synchronization of the cells. Surprisingly, it was discovered that the growth of Alex-κ cells was serum-independent compared to that of Alex cells (Figure 3A). Continuous Alex-κ cell growth was observed up to 5 days in the absence of fetal bovine serum. In contrast, after 48 h of serum starvation, growth arrest occurred in Alex cells. The cells were released from growth arrest by the addition of serum into the

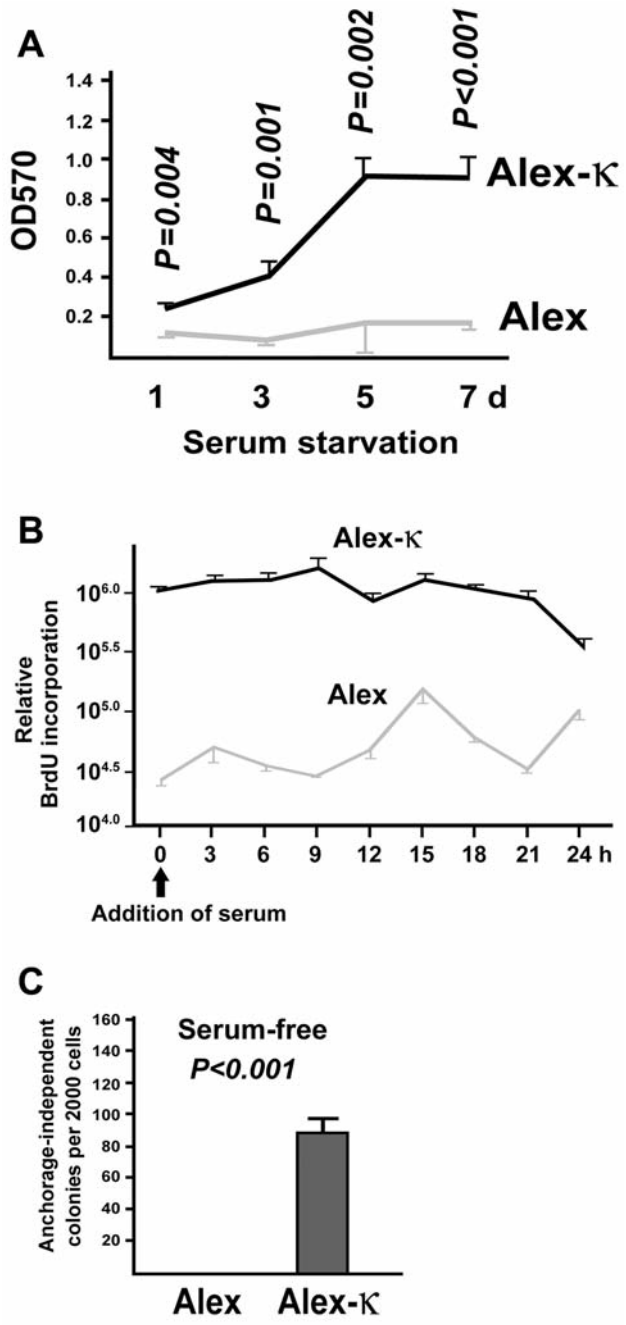

Figure 3. *Serum-independent growth of Alex-κ cells. A: MTT assays performed in the absence of serum. B: BrdU incorporation assays for Alex and Alex-κ cells. Serum starvation was performed for 48 h followed by the addition of 10% fetal bovine serum into the culture medium. BrdU incorporation was measured every 3 h. C: Efficiency of colony formation in soft agar in serum-free conditions.*

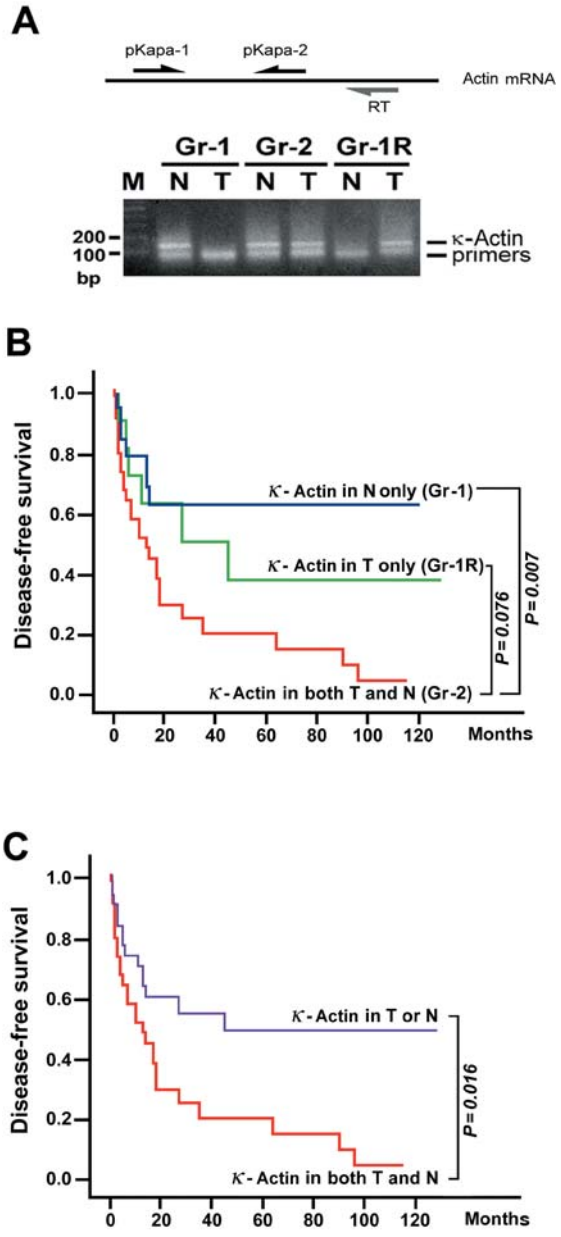

Figure 4. *Association between the κ-actin expression patterns and disease-free survival in HCC patients. A: Detection of κ-actin mRNA in noncancerous (N) and cancerous (T) HCC tissues using κ-actin-specific primers resulted in three expression patterns (Gr-1, Gr-2 and Gr-1R). B and C: Disease-free survivals of HCC patients with various κ-actin expression patterns.*

culture medium. In Alex cells, BrdU incorporation assays indicated that the cell cycle restarted in a small fraction of cells at 3 h after serum replenishment, while the majority of cells restarted their cell cycles 15 h after serum replenishment (Figure 3B). In contrast to Alex cells, a very high level of BrdU incorporation was detected in Alex-κ cells even after 48 h of serum starvation. The level of BrdU incorporation slowly decreased with time after the replenishment of serum. At 48 h after serum starvation, a significantly higher level of anchorage-independent growth was observed for Alex-κ cells (Figure 3C).

Table I. *Univariate and multivariate Cox proportional hazard analysis of clinicopathological parameters for disease-free survival in HCC patients.*

Parameter	No. of patients	Univariate		Multivariate	
		HR (95% CI)	*P*-Value	Adjusted HR (95% CI)	*P*-Value
Age (years)		1.006 (0.985-1.029)	0.574	0.985 (0.954-1.017)	0.350
Gender					
Female	17				
Male	55	1.087 (0.516-2.287)	0.827	0.505 (0.177-1.438)	0.201
Cirrhosis					
No	30				
Yes	42	1.756 (0.898-3.435)	0.100	2.517 (0.843-7.516)	0.098
Alcoholism					
No	46				
Yes	26	1.128 (0.601-2.115)	0.708	0.581 (0.202-1.667)	0.312
Tumor characteristics					
Microvascular invasion					
No	43				
Yes	27	1.922 (1.012-3.648)	0.046	2.061 (0.680-6.247)	0.201
Edmondson's grading					
I-II	41				
III-IV	31	1.806 (0.967-3.372)	0.064	1.520 (0.605-3.819)	0.373
Encapsulation					
No	21				
Yes	51	0.834 (0.434-1.604)	0.587	0.546 (0.202-1.476)	0.546
Tumor number					
1	43				
>1	29	2.662 (1.377-5.144)	0.004	4.561 (1.968-10.572)	<0.001
Largest tumor size (cm)		1.041 (0.987-1.099)	0.142	1.115 (0.975-1.274)	0.111
Macrovascular invasion					
No	65				
Yes	7	3.030 (1.255-7.315)	0.014	1.837 (0.496-6.805)	0.363
AFP (100 ng/ml)		1.001 (1.000-1.002)	0.033	1.002 (1.000-1.003)	0.046
Ascites					
No	66				
Yes	6	1.819 (0.547-6.051)	0.329	2.419 (0.366-15.978)	0.359
Albumin (g/dl)		0.866 (0.490-1.530)	0.621	1.240 (0.457-3.363)	0.672
Bilirubin (mg/dl)		1.199 (0.835-1.723)	0.325	1.152 (0.619-2.146)	0.655
Prothrombin time (s)		1.035 (0.885-1.211)	0.666	1.047 (0.776-1.413)	0.764
Creatinine (mg/dl)		1.439 (0.415-4.986)	0.566	2.206 (0.488-9.972)	0.304
AST (U/l)		1.002 (1.000-1.004)	0.098	1.002 (0.997-1.007)	0.461
ALT (U/l)		1.000 (0.998-1.003)	0.811	0.997 (0.993-1.002)	0.249
Anti-HCV					
No	60				
Yes	12	1.305 (0.595-2.862)	0.507	1.902 (0.235-15.390)	0.547
HBsAg					
No	19				
Yes	53	0.858 (0.426-1.728)	0.669	1.611 (0.256-10.154)	0.612
κ-Actin in both cancerous and noncancerous tissue					
No	34				
Yes	38	2.162 (1.123-4.164)	0.021	2.385 (1.316-4.320)	0.004

HR, Hazard ratio; CI, confidence interval; AFP, alpha-fetoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus; HBsAg, hepatitis B surface antigen.

Clinicopathological analysis. To understand the clinical relevance of κ-actin expression in HCC, 72 pairs of HCC tissues were submitted for κ-actin expression analysis. By use of κ-actin-specific primers, three groups of patients were identified (Figure 4A). The transcripts were detected in noncancerous tissues only in 21 patients (Gr-1), in cancerous tissues only in 13 patients (Gr-1R), and in both cancerous and noncancerous tissues in 38 patients (Gr-2). Kaplan-Meier analysis showed that the disease-free survival was shorter in Gr-2 patients when compared to Gr-1 (p=0.007)

and Gr-1R (p=0.076) patients (Figure 4B), and when compared to Gr-1 plus Gr-1R patients (p=0.016; Figure 4C). On the other hand, no correlation was found between the κ-actin expression pattern and overall survival.

Univariate and multivariate Cox proportional hazard analyses were performed to estimate the relationship between the clinicopathological parameters and disease-free survival in HCC patients (Table I). In univariate analysis, it was discovered that microvascular invasion (p=0.046), macrovascular invasion (p=0.014), tumor number (p=0.004), alpha-fetoprotein (p=0.033), and κ-actin in both cancerous and noncancerous tissue (p=0.021) were associated with disease-free survival. In multivariate analysis, after adjustment for all other confounding factors in the table, tumor number (p<0.001), alpha-fetoprotein (p=0.046) and κ-actin in both cancerous and noncancerous tissue (p=0.004) remained independent predictors for disease-free survival.

Discussion

Our previous study revealed a novel subgroup of actin sequences in HCCs (19). Phylogenetic analysis indicated that this subgroup of sequences was genetically distant from those of α-, β-, and γ-actin and these were thus named κ-actin. Although κ-actin sequences were isolated from HCC tissues, there remained a possibility that they originated from non-hepatocyte components in HCC. By analyzing the actin sequences in four hepatoma cell lines here, it was clearly demonstrated that the κ-actin sequences originated from hepatocytes.

In order to be preserved during the course of multiple-step hepatocarcinogenesis, the κ-actin must have conferred a growth advantage. The present data indicated that compared to mock-transfected Alex cells, expression of κ-actin reduced the growth rate and anchorage-independent colony formation of Alex-κ cells. However, the percentage of apoptosis in cells also decreased. The most striking growth advantage gain was that the proliferation of Alex-κ cells became serum-independent. The molecular mechanism for the serum-independent growth of Alex-κ cells remains unclear at this time. Comparison of the gene expression profiles between κ-actin-expressing cells and mock cells might be helpful in deciphering this puzzle. Serum-independent growth of cancer cells could be advantageous to HCC, since rapid tumor growth usually leads to insufficient nutrient supply. Some of the current strategies of anticancer treatment involve interruption of tumor vessels, such as transcatheter arterial chemoembolization and administration of antiangiogenesis agents (25, 26). It is possible that κ-actin-mediated mechanisms play an important role in tumor resistance against such treatments.

Clinicopathological analysis showed that patients with κ-actin detectable in both the noncancerous and cancerous liver tissues have significantly poorer disease-free survival compared to those with κ-actin detectable in either tissue only. It is generally accepted that multiple molecular changes are required in hepatocarcinogenesis. As a result, the adjacent non-cancerous tissues usually possess certain pre-cancerous features, presumably including increased expression of κ-actin. Such a phenotype was preserved in cancerous liver tissue (Figure 4, Gr-2). However, liver cancer may also develop through other mechanisms independently of κ-actin expression and could thus lead to no κ-actin expression being detectable in cancerous tissues (Gr-1). Conversely, κ-actin expression could also occur only when the tissue undergoes carcinogenesis and thus, might only be detectable in the cancerous tissues (Gr-1R). In the latter two situations, the chance of recurrent HCC tissues utilizing a κ-actin-mediated mechanism was smaller (compared to Gr-2) and thus was associated with a better prognosis.

In conclusion, a novel class of actin isoform named κ-actin was found in HCC. Alex cells overexpressing κ-actin acquired altered growth properties. Clinical analysis showed that HCC patients with κ-actin expression in both the cancerous and non-cancerous liver tissues had a poorer postoperative disease-free survival.

Acknowledgements

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