Hepatocellular carcinoma (HCC) is a common malignant tumor and many people in Japan as well as in the rest of the world suffer from HCC due to hepatitis B or C viral infection. As is known for other cancers (1), HCC shows a multistage process of tumor progression (2–4). A precancerous lesion called adenomatous hyperplasia, which is a small and hypercellular lesion, evolves from damaged liver tissue infected with hepatitis virus B or C. These lesions develop into early HCC, which exhibits as a vague nodular lesion that includes nodule-in-nodule type HCCs and their corresponding noncancerous liver tissues using an oligonucleotide array. We found several up-regulated genes involved in HCC progression. Among these genes, heat shock protein (HSP70) was shown to be a molecular marker of early HCC. Cyclase-associated protein 2 (CAP2) was also listed as an up-regulated gene in early HCC. Early HCC develops into a progressed form. Nodule-in-nodule type HCC (progressed HCC within early HCC) represents the transition from early to progressed HCC and is useful in the molecular genetic analysis of HCC progression during multistage carcinogenesis.

In our previous study (5), we compared the expression profiles among early and progressed components of seven nodule-in-nodule type HCCs and their corresponding noncancerous liver tissues using an oligonucleotide array. We found several up-regulated genes involved in HCC progression. Among these genes, heat shock protein (HSP70) was shown to be a molecular marker of early HCC. Cyclase-associated protein 2 (CAP2) was also listed as an up-regulated gene in early HCC.

CAP was originally identified from budding yeast as being located downstream of the ras gene (6, 7) and as being a factor associated with adenyl cyclase (8–10). CAP also binds monomeric actin and, therefore, also possesses a cytoskeletal function (11–15). However, only a few studies about CAP have previously been reported in mammalian cells (16) and no studies about CAP in human cancer have been published. In the present study, we have investigated the expression of CAP2 and proved its overexpression in multistage carcinogenesis in HCC.

Materials and Methods

Tissue samples. HCCs and corresponding noncancerous liver tissues were obtained from patients who underwent surgical resection at
National Cancer Center Hospital and Keio University Hospital, Japan between 1991 and 2001. We macroscopically separated HCCs and noncancerous lesions. Specimens were immediately frozen in liquid nitrogen and stored at −80°C until use. For immunohistochemical analysis, 72 HCCs (including 29 early HCCs, 10 nodule-in-nodule type HCCs, and 33 progressed HCCs) and 6 adenomatous hyperplasias were analyzed. The specimens were fixed in 10% formalin and embedded in paraffin. Paraffin sections were stained with H&E. Histologic diagnosis was done according to the WHO criteria (17). Informed consents were obtained from the patients and the Ethical Committee of the National Cancer Center Hospital and Keio University School of Medicine both approved the procedures.

**Real-time quantitative reverse transcription-PCR analysis.** For reverse transcription-PCR (RT-PCR) analysis, total RNA was isolated from the tissue samples by Isogen (Nippon Gene Co. Ltd., Toyama, Japan). All RNA samples were treated with DNase I (Promega Corp., Madison, WI) to remove genomic DNA. Real-time quantitative RT-PCR analysis was done as previously reported (18). The primer set 5′-CTCCCAG-CAAAAGTCACACTCC-3′ (forward) and 5′-CTGACTCGACTTTTGTT-CAACCGA-3′ (reverse) was designed against the 3′-untranslated region of CAP2. Additionally, we used another primer set, 5′-CTCAGCTTTATTTCGCCCAGCTCA-3′ (forward) and 5′-CCCTCAGGCTGGGATTCTTGTA-3′ (reverse). For standardization of the amount of RNA, expression of glyceradehyde-3-phosphate dehydrogenase (GAPDH) in each sample was quantified by using the primer set 5′-GAAGGTGAAGGTCGGAGTC-3′ (forward) and 5′-CCCGAATCACATTCTCCAGAA-3′ (reverse). All PCR reactions were done with the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems, Foster City, CA) under the following conditions: 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Real-time detection of the emission intensity of SYBR Green was done with ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems) as previously reported (19). Quantitative RT-PCR was done at least thrice, including a no-template control as a negative control. Statistical analyses were done with the paired t test.

**Antibodies.** Polyclonal antibodies against CAP2 were generated in rabbits. The animals were immunized by using a synthetic peptide corresponding to the amino acid sequence of the COOH-terminal domain of human CAP2 (residues 460-475, KTAWDGSKLITEPAEI-C) as an antigen according to the methods previously described (10). Specificity of the antibody was further examined by an absorption test of the antibody with the antigen peptide and by immunoblotting and immunohistochemistry using five HCC cell lines (Li7, KYN-2, PLC/PRF/5, Kim-1, and HepG2) and HCC specimens (20).

**Immunoblotting.** For immunoblotting of CAP2, tissue samples and cells from HCC cell lines were homogenized on ice in 1 mL of lysis buffer.
buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L CaCl₂, and 0.05% Brij35 containing a cocktail of proteinase inhibitors; Roche Diagnostics GmbH, Basel, Switzerland]. After determining protein concentrations by the dye-binding method (Proteostain Protein Quantification Kit-Wide Range, Dojindo Laboratories, Kumamoto, Japan), the supernatants of the homogenates were subjected to SDS-PAGE (4.12% graduated gel; Invitrogen, Carlsbad, CA) under reduction. After blocking nonspecific reactions with 0.5% skim milk, the resolved proteins were transferred onto polyvinylidence difluoride membranes that had been incubated with rabbit polyclonal anti-CAP2 antibody. The membranes were incubated with horseradish peroxidase–conjugated antirabbit antibodies (1:20,000 dilution; Chemicon International, Inc., Temecula, CA) for CAP2. Immunoreactive protein bands were detected with enhanced chemiluminescence immunoblotting reagents (GE Healthcare, London, United Kingdom).

**Immunohistochemistry.** Immunohistochemical staining was done on formalin-fixed, paraffin-embedded tissue sections with the simple stain technique with an anti-CAP2 antibody (dilution, 1:800). For staining, deparaffinized and rehydrated sections were heated at 120°C in 0.01 mol/L sodium citrate buffer for 10 minutes before incubation with the antibody. Three pathologists evaluated the tissue sections and staining and CAP2 positivity was expressed as a percentage of positive tumor cells in each lesion.

**Results**

**CAP2 mRNA expression in HCC.** CAP2 was one of the upregulated genes detected in the early component of nodule-in-nodule type HCC (5). To confirm our findings, we analyzed the level of CAP2 mRNA in nodule-in-nodule type HCCs by real-time quantitative RT-PCR. In six of seven cases, the expression level in the early component was up-regulated compared with that in the corresponding early component (Fig. 1A and B). The average expression level of CAP2 mRNA in nodule-in-nodule type HCC was up-regulated in a stepwise manner (i.e., noncancerous liver (1.26 ± 0.65) versus early component (2.89 ± 1.56), P = 0.001; early component versus progressed component (5.20 ± 3.71), P = 0.041). Additionally, we analyzed the level of CAP2 mRNA in eight cases of HCCs by using another set of controls, which crossed a splice junction (Fig. 1C and D). Two of eight cases were well-differentiated HCCs, three cases were moderately differentiated HCCs, and three cases were poorly differentiated HCCs. The average expression level of CAP2 mRNA was significantly higher in HCC than in noncancerous liver (1.142 ± 0.996 versus 0.292 ± 0.455; P < 0.001).

**Protein expression of CAP2.** To determine whether CAP2 was also overexpressed at the protein level, we raised a polyclonal antibody against CAP2. Using this antibody, we examined the HCC cell lines and HCC tissues, as well as noncancerous liver tissues, by Western blot analysis. A single 53-kDa band of strong intensity was observed in all the HCC specimens. Lane 1, Li-7; lane 2, KYN-2; lane 3, PLC/PRF/5; lane 4, Kim-1; lane 5, HepG2; Lane 6, CaCl₂, and 0.05% Brij35 containing a cocktail of proteinase inhibitors; buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L CaCl₂, and 0.05% Brij35 containing a cocktail of proteinase inhibitors; Roche Diagnostics GmbH, Basel, Switzerland]. After determining protein concentrations by the dye-binding method (Proteostain Protein Quantification Kit-Wide Range, Dojindo Laboratories, Kumamoto, Japan), the supernatants of the homogenates were subjected to SDS-PAGE (4.12% graduated gel; Invitrogen, Carlsbad, CA) under reduction. After blocking nonspecific reactions with 0.5% skim milk, the resolved proteins were transferred onto polyvinylidence difluoride membranes that had been incubated with rabbit polyclonal anti-CAP2 antibody. The membranes were incubated with horseradish peroxidase–conjugated antirabbit antibodies (1:20,000 dilution; Chemicon International, Inc., Temecula, CA) for CAP2. Immunoreactive protein bands were detected with enhanced chemiluminescence immunoblotting reagents (GE Healthcare, London, United Kingdom).

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**Table 1. CAP2 expression in HCCs and precancerous lesions**

<table>
<thead>
<tr>
<th>Histology</th>
<th>CAP2 positivity (%)</th>
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<tbody>
<tr>
<td></td>
<td>5-50</td>
<td>50-70</td>
</tr>
<tr>
<td>Adenomatous hyperplasia</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Early HCC</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Progressed HCC</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Nodule-in-nodule type HCC</td>
<td></td>
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<tr>
<td>Early component</td>
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<td>1</td>
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<td>Progressed component</td>
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alwals stronger in progressed components than in early components. In progressed HCC, all except one case were positive for CAP2 and 28 cases showed 70% to 100% positivity, which was diffuse and strong. CAP2 expression was observed in the cytoplasmsof HCC and it was also observed in the membranous or apical portions of progressed HCC in pseudoglandular patterns (Fig. 4C and D). Immunoreactivity was remarkably reduced to a negligible level by incubating the CAP2 antibody with the antigen peptide in an absorption test (data not shown).

Fig. 3. Immunohistochemical analysis of CAP2. A, damaged liver (liver cirrhosis); C, adenomatous hyperplasia (AH); E, early HCC; G, nodule-in-nodule type HCC (H&E stain). B, D, F, and H, corresponding CAP2 immunostaining of each serial section. Arrowheads, borders between adenomatous hyperplasia, early HCC, and noncancerous liver. Smooth muscle of the vascular wall served as the positive control. B, the periphery of regenerative nodules of liver cirrhosis was focally positive for CAP2. D, adenomatous hyperplasia was negative for CAP2. H, early HCC and progressed HCC were positive for CAP2. In nodule-in-nodule type HCC, the progressed HCC component was more strongly positive than the early HCC component.
Discussion

In this study, we found that CAP2 was overexpressed at the mRNA and protein level in early and progressed HCC. Neo et al. (21) identified several genes that were up-regulated in human HCCs using gene expression profiling. CAP2 was listed as one of the overexpressed genes; however, it was not further analyzed in the article. This is the first report to indicate that CAP2 shows overexpression in multistage hepatocarcinogenesis as well as in human cancer.

The differential diagnosis of early HCC from precancerous lesions or noncancerous liver tissue is difficult for pathologists because of the very well differentiated histology with minimum atypia in early HCC. Histologic diagnosis is sometimes very difficult, and thus molecular markers are considered to be important to make precise diagnoses and to administer appropriate treatments. Although the periphery of the regenerative nodules of liver cirrhosis and adenomatous hyperplasias were positive for CAP2 focally and weakly, when tumor cells show strong and wide positivity, the nodule could be diagnosed as HCC. Further extensive analysis will show if CAP2 could be a sensitive and practical molecular marker when pathologists make a diagnosis of early HCC.

The molecular changes that occur in early HCC are not well understood. Early HCC is characterized by an increase in cell density and growth. HSP70 is the only positive molecular marker identified thus far (5). We previously suggested that a stressful environment in early HCC might stimulate HSP70 synthesis (5). CAP is involved in regulating the adenyl cyclase activity in yeast under the control of ras (6–8). Noegel et al. (10) reported an interaction of CAP with adenylyl cyclase in Dictyostelium and an influence on signaling pathways directly as well as through its function as a regulatory component of the cytoskeleton. Previous studies have shown that humans and rats have two different CAP proteins (22, 23). However, only a few studies on CAP have been done in mammalian cells. Overexpression of CAP2, which is located downstream of ras, might be associated with hepatocarcinogenesis where a mutation of ras has not been detected in human HCC. Cherkasova et al. (24) reported that Kss1 mitogen-activated protein kinase cascades acted upstream of the ras/cyclic AMP pathway to regulate survival. In yeast, the major function of Kss1 mitogen-activated protein kinase cascade is to activate adenylyl cyclase and to control cell proliferation (25, 26). This functional link between mitogen-activated protein kinase and cyclic AMP might be related to proliferative activity and carcinogenesis through CAP2 overexpression in HCC.

CAP2 is a striated muscle-specific protein expressed during early mouse development. At later developmental stages, CAP2 also becomes strongly expressed in specific areas of the central nervous system, and in adult mice, expression is especially strong in the heart, skeletal muscle, and brain (27). Bertling et al. (27) reported that CAP1 promotes rapid actin dynamics in conjunction with ADF/cofilin and is required for several central cellular processes in mammals. Kang and Jiang (14) reported that CAP2 is one of the genes that encode regulators of the actin cytoskeleton and cell polarity. Yusof et al. (15) reported that CAP possesses actin binding activity. In the present study, CAP2 was up-regulated in early HCC and even greater overexpression was observed in progressed HCC. The ability of stromal invasion to occur in early HCC might be related to the actin...
binding activity of CAP. CAP2 is also more strongly expressed in apical portions in a pseudoglandular pattern (Fig. 4C and D). It might also be related to the location of actin in tumor cells. We are now investigating the relationship between CAP2 and actin binding activity. CAP2 is rarely expressed in tumor cells. We are now investigating the relationship between cell lines will be required to identify the cellular roles of CAP2 in hepatocarcinogenesis. Further investigation with human HCC cells will be required to identify the cellular roles of CAP2 in the progression of HCC.

In conclusion, we present the first indication that CAP2 is overexpressed in human cancer. CAP2 was overexpressed in early HCC when compared with noncancerous liver tissue and precancerous lesions. The higher overexpression of CAP2 observed in progressed HCC, when compared with early HCC, is suggestive of an involvement of this protein in multistep carcinogenesis.

Acknowledgments

We thank Drs. T. Hibi and T. Mamiya for providing samples and clinical data and M. Fujiwara and M. Morioka for technical assistance.

References

2. Tsuda H, Hirohashi S, Shimosato Y, Terada M. HCC, is suggestive of an involvement of this protein in multistep carcinogenesis. Further investigation with human HCC cell lines will be required to identify the cellular roles of CAP2 in the progression of HCC.

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References

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