Apg-2 has a chaperone-like activity similar to Hsp110 and is overexpressed in hepatocellular carcinomas

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Received 4 November 2003; revised 16 December 2003; accepted 17 December 2003

First published online 29 January 2004

Edited by Jesus Avila

Abstract Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. We constructed subtracted cDNA libraries enriched with genes overexpressed in HCCs. Among the 17 genes identified were molecular chaperones, Hsp110, Hsp90B, and Hsp70-1. Expression of the Hsp110 family members was further analyzed, and increased transcript levels of Hsp110 and Apg-2, but not Apg-1, were found in 12 and 14, respectively, of 18 HCCs. Immunohistochemical analysis demonstrated the overexpression of the proteins in tumor cells. Apg-2 had chaperone ability similar to Hsp110 in a thermal denaturation assay using luciferase, and showed anti-apoptotic activity. These results suggest that the Hsp110 family members play important roles in hepatocarcinogenesis through their chaperoning activities.

Key words: Hepatocellular carcinoma; Hsp70; Hsp110; Apg-2; Apg-1; Chaperone

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in Asia and Africa, where hepatitis virus infections and exposure to specific liver carcinogens are prevalent [1,2]. Although screening of high risk populations by ultrasonography and measurement of the serum α-fetoprotein level has facilitated the early detection of HCC, the overall survival of patients with HCC is unsatisfactory. Several studies have identified alterations of several genes, but the molecular mechanisms of hepatocarcinogenesis have not been fully elucidated [1,2]. It is, therefore, important to identify molecules that can be used as diagnostic or prognostic markers and molecular targets to develop novel preventive or therapeutic agents.

A number of conditions that are damaging to cellular proteins can trigger the increased expression of several highly conserved proteins that are referred to as heat shock proteins (Hsps)[3,4]. Hsps not only protect cells from the toxic effects of heat and other stresses, but also have multiple housekeeping functions, such as folding and translocating newly synthesized proteins, activation of specific regulatory proteins, protein degradation, protein signaling and antigen presentation. Additionally, Hsps can inhibit the irreversible aggregation of denatured proteins and, in some instances, function in the refolding of denatured proteins. The major classes of mammalian Hsps include Hsp110s, Hsp90s, Hsp70s, Hsp60s, Hsp40s and the small Hsps [5].

Hsp110 cDNAs were initially cloned from Chinese hamster and mouse (often called Hsp105) [6,7]. Subsequently, we have isolated Hsp110-related genes, Apg (ATP and peptide-binding protein in germ cells)-1/Osp94 and Apg-2, and demonstrated that the human as well as mouse Hsp110 family consisted of Apg-1, Apg-2 and Hsp110 [8–10]. Constitutive expression of Apg-1 is mainly observed in the testis, while expression of Hsp110 and Apg-2 is observed in various organs [11]. Hsp110 has been identified as one of the genes induced by the human papilloma virus oncoprotein E7 [12]. Of the three members of the Hsp110 family, expression of Hsp110 and Apg-1, but not Apg-2 is inducible by heat stress in mouse and human cells [8,10]. In contrast, expression of all Hsp110 family members is induced after transient forebrain ischemia in rats [13]. Collectively, these observations suggest that the expression of each Hsp110 family member is independently regulated and inducible under various conditions including oncogenic stress.

By constructing subtracted cDNA libraries enriched with the genes overexpressed in HCCs, we have previously reported identification of the novel proteins gankyrin and HSCO [14,15]. Gankyrin is overexpressed in most HCCs and accelerates the degradation of the retinoblastoma gene product (Rb). HSCO shows anti-apoptotic activity and enhances nuclear export of NF-κB. In this study, we used the remaining cDNA clones as probes in Northern blot analysis, and identified 17 genes overexpressed in HCCs including three molecular chaperones. We further examined expression of all members of the Hsp110 family in HCCs, and determined the molecular chaperoning activity of Apg-2 in vitro and in vivo.

2. Materials and methods

2.1. Surgical specimens

Primary HCCs and corresponding non-malignant liver tissue were obtained from 18 patients undergoing surgical resection at the Kyoto University Hospital, Japan. Informed consent was obtained from each patient. The stage of tumor progression was determined by serum tumor marker, CT scan, chest X-ray and pathological analysis. The

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Abbreviations: HA, hemagglutinin; HCC, hepatocellular carcinoma; Hsp, heat shock protein; Ig, immunoglobulin; PBS, phosphate-buffered saline; Rb, retinoblastoma gene product

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00034-1

age of the patients was 62.1 ± 8.5 years (mean ± S.D.). Twelve were males and six were females. There were three, six, seven and two patients with stage I, II, III and IV HCCs, respectively. The degree of tumor cell differentiation was assessed according to the classification of Edmondson and Steiner. Six patients were positive for the hepatitis B surface antigen and/or antibody to it, and 11 were positive for the anti-hepatitis C antibody. The relationship between Hsp110 or Apg-2 expression and clinicopathological status was analyzed by Fisher's exact probability two-tailed test using the JMP software (SAS Institute, Cary, NC, USA).

2.2. Cell culture

Human 293T cells, mouse NIH/3T3 cells, monkey COS7 cells and their transfectants were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C, 5% CO_2 in air. Transfection and isolation of stable clones were performed as described [14]. NIH/3T3 Tet-On gankyrin cells were made by co-transfection of NIH/3T3 cells with pTet-On plasmid and pTRE2 plasmid (Clontech, Tokyo, Japan) containing gankyrin cDNA, followed by selection of clones inducibly overexpressing gankyrin. The numbers of viable cells were counted by the trypan blue exclusion method using a hemocytometer under a microscope.

2.3. Construction of subtracted cDNA libraries

Isolation of RNA and the cDNA subtraction were performed essentially as described previously [14,16]. Total RNA was extracted from two stage IV HCCs and their corresponding non-malignant tissues. After synthesizing double-stranded cDNAs, we constructed subtracted cDNA libraries by using our original method [16] or with the PCR Select Subtraction kit (Clontech, Palo Alto, CA, USA) following the manufacturer's protocol.

2.4. Antibodies

Polyclonal antibodies were raised by immunizing rabbits with glutathione *S*-transferase fusion proteins of the mouse Apg-1 or Apg-2 corresponding to amino acids 96–582 or 60–841, respectively. A polyclonal anti-Hsp110 antibody was a kind gift from Dr. H. Itoh, Akita University [17]. Affinity-purified immunoglobulin (Ig) derived from preimmune rabbit sera was used as a negative control. Mouse monoclonal anti-hemagglutinin (HA) antibody (Boehringer Mannheim Biochemica, Mannheim, Germany), anti-His antibody (Clontech), anti-gankyrin antibody [14] and horseradish peroxidase-conjugated goat anti-rabbit Ig (Bio-Rad, Hercules, CA, USA) were diluted in phosphate-buffered saline (PBS) containing 5% bovine serum albumin to the appropriate concentrations.

2.5. Analysis of gene expression

Northern and Western blot analyses were performed as described [15]. Specificity of the cDNA probe used for each Hsp110 family member has been verified [10]. The band intensities were compared between the HCC and non-cancerous tissues from the same patient after normalization. To determine the specificity of antibodies, cDNAs containing full coding regions for human Hsp110, Apg-1 and Apg-2 were cloned into pCAGGS vector with HA tags (pCMV-3HA). 293T cells were transfected with these plasmids or vector alone to express Hsp110 family members N-terminally tagged with HA or HA alone, respectively, and cell lysates were subjected to Western blotting.

Immunohistochemistry was performed as described [15] using sections from the tissues fixed in 4% paraformaldehyde in PBS and embedded in wax. Peroxidase-conjugated anti-rabbit IgG and diaminobenzidine were used to visualize the bound antibodies. As a control, the preimmune serum was used at the same dilution.

2.6. Chaperone activities

Apg-2 cDNA was cloned into pQE9 vector (Qiagen, Tokyo, Japan), and chaperone activities were assayed as previously described [18]. Briefly, 50 nM luciferase (Sigma Aldrich, Tokyo, Japan) was equilibrated to room temperature with ovalbumin or recombinant Apg-2, followed by incubation at 43°C in a thermostated cuvette. Light scattering by protein aggregation was determined by measuring the increase in optical density at 320 nm with a spectrophotometer. For luciferase reactivation experiments, luciferase (150 nM) was incubated with ovalbumin or recombinant Apg-2 at 43°C for 30 min and diluted to 15 nM in 60% rabbit reticulocyte lysate (Promega) at 30°C. For the measurement of luciferase activity, the reactivation solution was five-fold diluted, and added to luciferase assay solution (Promega). The activity was measured with a Lumat LB 9501 luminometer (Berthold).

In vivo chaperoning activity of Apg-2 was examined by co-transfecting COS7 cells with plasmids expressing mutant HSCO fused with green fluorescent protein (GFP) (H. Higashitsuji, unpublished observation) and plasmids expressing Apg-2, Hsp70 or vector alone.

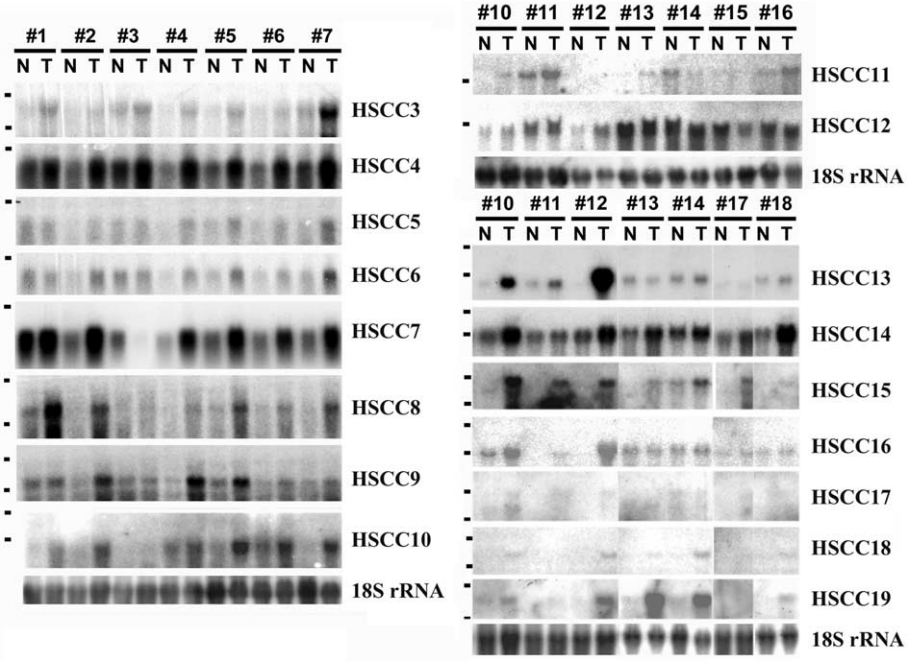

Fig. 1. Increased expression of genes isolated from hepatoma-derived subtracted cDNA libraries in HCCs. Ten μg of total RNA from pairs of non-cancerous liver tissue (N) and HCC (T) were subjected to Northern blot analysis using ^{32}P-labeled hepatoma-derived subtracted cDNA clones (HSCC3 to HSCC19) as probes as indicated. cDNA for 18S ribosomal RNA (rRNA) was used as a control. Mobilities of 18S and/or 28S rRNAs are indicated on the left. Case numbers are shown at the top.

Thirty-six hours after transfection, the percentage of cells with cytoplasmic aggregates in GFP-positive cells was determined with a confocal laser microscope (Olympus, Tokyo, Japan). The percentage of apoptotic cells was also assessed by determining the percentage of cells with sub-G1 DNA content by flow cytometry after staining with propidium iodide.

3. Results

3.1. Identification of genes overexpressed in HCCs

After analyzing the subtracted cDNA libraries enriched with genes overexpressed in HCCs compared with non-cancerous liver tissues, we identified 17 distinct cDNA clones in addition to those of HSCO and gankyrin previously reported [14,15]. Their expression was increased in at least two out of seven HCCs analyzed by Northern blotting (Fig. 1). A homology search with BLAST software revealed that all cDNAs corresponded to known genes including *Hsp110*, *Hsp90B* and *Hsp70-1* (Table 1).

3.2. Increased expression of Hsp110 family members in HCCs

Since the human Hsp110 family consists of Hsp110, Apg-1 and Apg-2 [10], we further examined expression of Apg-1 and Apg-2 as well as Hsp110 in a total of 18 HCCs. The transcript level of Hsp110 was increased in 12 of 18 HCCs (Figs. 1 and 2). The transcript level of Apg-2 was increased in 14 of 18 HCCs (Fig. 2). All 12 HCCs overexpressing Hsp110 also overexpressed Apg-2. In contrast, no expression of Apg-1 was detected in HCCs or the non-cancerous liver tissues (data not shown). The relationship between the overexpression of Hsp110 or Apg-2 and the clinicopathological findings was examined, but no significant association with virus infection status, α-fetoprotein level, histologic grade, portal involvement, number of tumors, or clinical stage was identified (data not shown).

Human papilloma virus oncoprotein E7 is known to induce Hsp110 [12]. Since gankyrin, which is commonly overexpressed in HCCs, has activities similar to E7 [14], we investigated the possibility that gankyrin might induce expression of Hsp110 or Apg-2. When gankyrin was overexpressed in NIH/3T3 cells, the levels of Hsp110 and Apg-2 transcripts were not increased, suggesting that gankyrin is not related to the overexpression of Hsp110 and Apg-2 (data not shown).

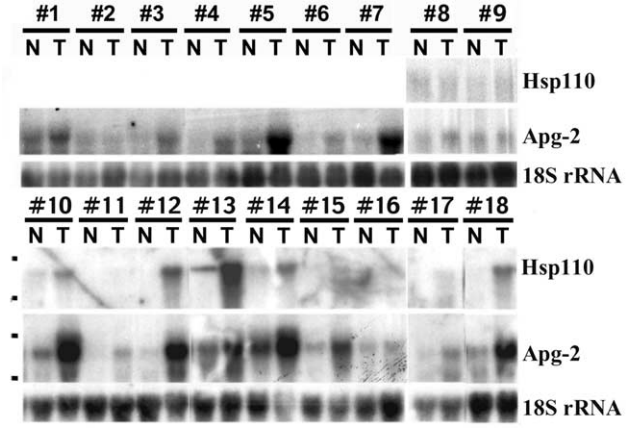

Fig. 2. Increased transcript levels of the Hsp110 family members in HCCs. Ten μg of total RNA from pairs of non-cancerous liver tissues (N) and HCCs (T) were subjected to Northern blot analysis using cDNA for Hsp110 or Apg-2 as probes as indicated. cDNA for 18S ribosomal RNA (rRNA) was used as a control. Mobilities of 18S and 28S rRNAs are indicated on the left. Case numbers are shown at the top.

3.3. Increased expression of Hsp110 and Apg-2 proteins in HCCs

We next examined the protein levels of Hsp110 and Apg-2 in HCCs. Since the amino acid sequences of Hsp110, Apg-1 and Apg-2 are quite similar to each other and between mice and humans, we first determined the specificity of polyclonal antibodies raised against the respective mouse proteins. As shown in Fig. 3, the mobilities of HA-tagged Hsp110 and HA-tagged Apg-2 were slower than that of HA-tagged Apg-1. The anti-Hsp110 antibody recognized Hsp110 and Apg-1. The anti-Apg-1 antibody recognized Apg-1, but not Apg-2 or Hsp110. The anti-Apg-2 antibody specifically recognized Apg-2. Since no expression of Apg-1 was detected in HCCs by Northern blot analysis, the anti-Hsp110 antibody was used to determine the protein level of Hsp110. In three pairs of

Table 1
Identity of clones isolated by subtractive hybridization

Clone	Identity (accession number)	Function
HSCC1[a]	HSCO (hepatoma subtracted cDNA clone one) (D83198)	anti-apoptosis
HSCC2[a]	Gankyrin (PSMD10, p28) (D83197)	increased degradation of Rb
HSCC3	Heat shock 105/110 kDa protein 1 (HSPH1, HSP105B, HSP110) (NM_006644)	chaperone
HSCC4	Ribosomal protein L14 (RPL14) (NM_003973)	translation
HSCC5	Ribosomal protein S17 (RPS17) (NM_001021)	translation
HSCC6	Ribosomal protein S15a (RPS15A) (NM_001019)	translation
HSCC7	Apolipoprotein A_2 (APOA2) (NM_001643)	lipid metabolism
HSCC8	Cathepsin F (CTSF) (NM_003793)	lysosomal cysteine protease
HSCC9	ADP-ribosylation factor-like 6 interacting protein-1 (Aip-1) (XM_027365)	unknown, binding to ARL-6 in yeast
HSCC10	Hypothetical protein MGC2668 (NM_030914)	unknown, ubiquitin-related modifier-like
HSCC11	Fatty acid coenzyme A ligase, long-chain 5 (FACL5) (NM_016234)	fatty acid metabolism
HSCC12	Pancreatitis-associated protein (PAP, PAP1, HIP) (NM_002580)	C-type lectin
HSCC13	Ferritin, light polypeptide (FTL) (NM_000146)	iron storage
HSCC14	Elongation factor 1-α1 (EEF1A1) (NM_001402)	translation
HSCC15	Plasmalemma vesicle-associated protein (PLVAP, PV-1) (NM_031310)	endothelial membrane protein
HSCC16	KIAA1274 (AB033100)	unknown, PTPase catalytic domain-like
HSCC17	SPARC-like 1 protein (SPARCL1, mast9, hevin) (NM_004684)	anti-adhesive extracellular matrix protein
HSCC18	Heat shock 90 kDa protein 1, β (HSPCB, HSP90B, HSPC2) (NM_007355)	chaperone
HSCC19	Heat shock 70 kDa protein 1A (HSPA1A, HSP70-1) (NM_005345)	chaperone

[a]Previously reported [14,15].

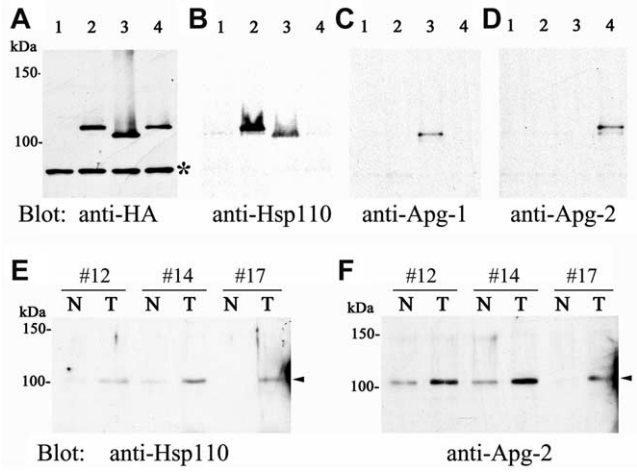

Fig. 3. Increased protein levels of Hsp110 and Apg-2 in HCCs. A–D: Specificity of the antibodies. Cell lysates from 293T cells transfected with plasmids expressing HA alone (lanes 1), and Hsp110 (lanes 2), Apg-1 (lanes 3) or Apg-2 (lanes 4) N-terminally tagged with HA were Western-blotted and probed with a monoclonal mouse anti-HA antibody (A), polyclonal rabbit anti-Hsp110 antibody (B), anti-Apg-1 antibody (C) or anti-Apg-2 antibody (D). Asterisks indicate non-specific bands. E,F: Overexpression of Hsp110 and Apg-2 proteins. Total extracts from three pairs of non-cancerous liver tissues (N) and HCCs (T) were Western-blotted and probed with the indicated antibodies. Arrowheads indicate the mobilities of the specific proteins.

HCC specimens analyzed, both Hsp110 and Apg-2 protein levels were increased in HCCs compared with the non-cancerous tissues (Fig. 3E,F), and the results were consistent with those of the Northern blot analysis.

To identify the cells overexpressing Hsp110 and Apg-2 in HCC tissues, we performed an immunohistochemical analysis (Fig. 4). Specific signals for Hsp110 and Apg-2 were observed in the cytoplasm of HCC cells, but not in the adjacent non-cancerous cells, demonstrating that the expression of Hsp110 and Apg-2 proteins was increased in cancer cells.

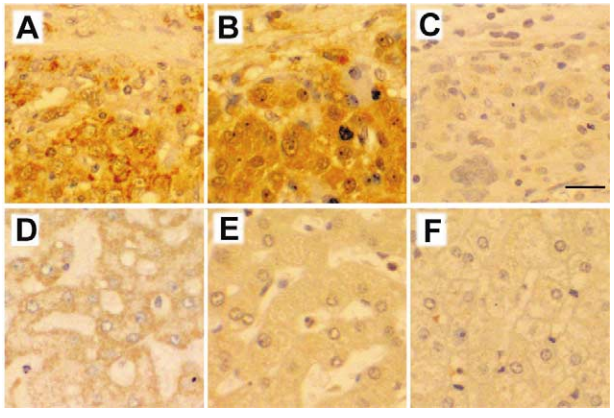

Fig. 4. Immunohistochemical analysis of expression patterns of Hsp110 and Apg-2 in HCCs. Tissue sections were made from HCC (A–C) and non-cancerous liver tissue (D–F). Immunohistochemical staining was performed using an anti-Hsp110 antibody (A,D), anti-Apg-2 antibody (B,E) or preimmune serum (C,F). Note, top areas of panels A–C are non-cancerous tissues and are not stained with either the anti-Hsp110 or anti-Apg-2 antibody. Bar, 50 μm.

3.4. Chaperoning activity of Apg-2

The chaperoning activities of Hsp110 and Apg-1 have been demonstrated [17,18]. To determine whether Apg-2 has equivalent chaperoning activity, we used luciferase as a reporter in basic holding and folding assays. As shown in Fig. 5A, Apg-2 was efficient in inhibiting the heat-induced aggregation of luciferase in vitro. When Apg-2 was present during heating at a 1:1 molar ratio with luciferase, the enzyme remained highly soluble.

When the activity of luciferase heated in the presence of Apg-2 was measured, however, no original activity was observed nor was activity restored during further incubation of the complex (data not shown). To determine whether Apg-2 requires the cooperation of other Hsps and/or chaperones to refold denatured substrate proteins, we heated luciferase in the presence of Apg-2 or ovalbumin, and then added the proteins to a 60% rabbit reticulocyte lysate that has been shown to be an optimal refolding medium [18]. As shown in Fig. 5B, luciferase heated in the presence of Apg-2 regained 46% of the original activity, whereas luciferase heated with ovalbumin did not. These results demonstrate that Apg-2 is a potent chaperone in inhibiting aggregation and maintaining

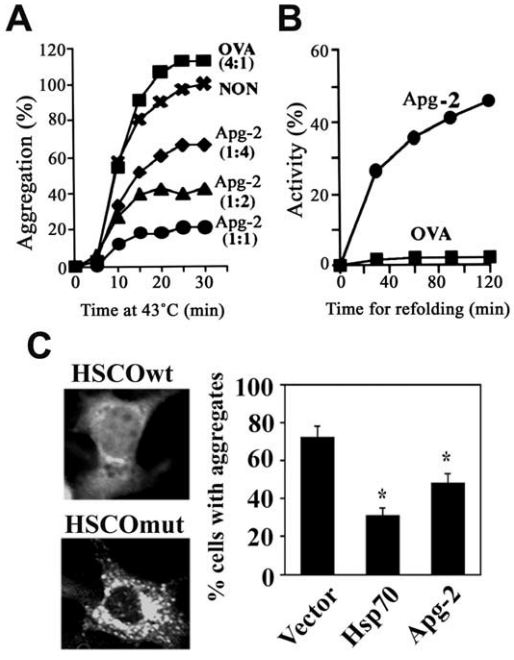

Fig. 5. Chaperone activity of Apg-2. A: Effects on protein aggregation in vitro. Heat-induced aggregation of luciferase in the presence or absence of Apg-2 was assayed by light scattering. Ovalbumin (OVA) was used as a control protein. Molar ratios to luciferase are indicated in parentheses. The values are expressed as percentage of the aggregation observed when luciferase alone (NON) was incubated for 30 min at 43°C. B: Effects on protein refolding in vitro. Luciferase was heated for 30 min at 43°C in the presence of a 20-fold molar excess of Apg-2 or OVA. The proteins were then added to 60% rabbit reticulocyte lysate, allowed to refold at 30°C for the indicated times, and the luciferase activity was measured as described [18]. C: Effects on aggregation of mutant proteins in vivo. COS7 cells were transfected with plasmids expressing wild-type HSCO or mutant HSCO fused with GFP (HSCOwt and HSCOmut, respectively) and observed under a fluorescence microscope (left). COS7 cells were co-transfected with HSCOmut plasmids and plasmids expressing Hsp70, Apg-2, or vector alone as indicated, and the percentage of cells with cytoplasmic aggregates in GFP-positive cells was determined (right). $*P < 0.05$ vs. vector alone.

a folding-competent state but is incapable of refolding heat-denatured proteins on its own.

To determine the chaperoning activity of Apg-2 in vivo, we utilized mutant HSCO which forms cytoplasmic aggregates and induces apoptosis when expressed in COS7 cells (H. Higashitsuji, unpublished observation). As shown in Fig. 5C, expression of Apg-2 significantly reduced the formation of aggregates. At the same time, the percentage of cells with sub-G1 DNA content was reduced compared with control ($21.2 \pm 1.9\%$ vs. $27.6 \pm 1.9\%$, $P < 0.05$).

4. Discussion

One approach to understand the molecular mechanisms of carcinogenesis, progression and drug resistance is to compare gene expression between tumor tissues and normal tissues. By using microarrays, Okabe et al. [19] have analyzed expression patterns of 24 030 genes in 20 HCCs, and suggested that expression of specific genes could be associated with the viral etiology of the tumor, vascular invasion and other features. Chen et al. [20] have analyzed 17 400 genes in 82 HCCs, and found 1640 genes to be up- or down-regulated. Recently, Chuma et al. [21] have found that Hsp70 is a marker of early HCCs. In this and our previous studies [14,15], we have utilized the cDNA subtraction method followed by Northern blotting, and identified 19 genes overexpressed in HCCs. Interestingly, two of them were novel genes and six have not been identified to be overexpressed in HCCs by the microarray technique [19–25].

Hsps have been suggested to play roles in tumorigenesis as modifiers of protein activities, in particular, components of the cell cycle machinery, kinases, and other proteins implicated in cancer progression [4,26]. Overexpression of Hsp70 has been correlated with advanced stages, poor prognosis or resistance to therapy in breast cancers, endometrial cervix cancers and HCCs [21,26,27]. However, in osteosarcomas, the overexpression of Hsp70 has been associated with response to chemotherapy [28]. Overexpression of Hsp27 has been correlated with high histologic grade and poor prognosis in HCCs, but with improved prognosis in others [27,29]. These inconsistent findings suggest that Hsps may have a pivotal function in cell proliferation depending on tumor type. In HCCs, we found that transcripts for Hsp110 family members, Hsp110 and Apg-2, were overexpressed in 67% and 78%, respectively, of HCC tissues, and that the tumor cells expressed these proteins. Although Hsp110 functionally interacts with Hsc70 and Hsp27 [30] which are prognostic indicators in some cancer types, the increased expression of Hsp110 and Apg-2 did not correlate with any clinical findings.

Molecular chaperones induced in response to stress appear to function at key regulatory points in the control of cell survival. For example, overexpression of Hsp70 protects tumor cells from a variety of stresses [26]. Hsp110 as well as Hsp70 confers thermal tolerance to cells [18]. The protective function of the Hsps may be extended to include an anti-apoptotic role for several members of the Hsp family including Hsp90, Hsp70 and Hsp27 [26,31]. Furthermore, chaperones have been shown to repair the conformational defects of some mutated proteins, thus reducing their phenotypic effects [32]. In this study, we demonstrated the in vitro chaperoning activity of Apg-2, and showed that Apg-2 decreases aggregate formation and apoptosis induced by mutant HSCO proteins. Since evasion of apoptosis is one of the hallmarks of the cancer cell phenotype, Hsp110 family members probably contribute to hepatocarcinogenesis by acting as molecular chaperones in cooperation with other Hsps to inhibit apoptotic pathways induced by mutant proteins. Further studies on the functions of Hsp110 and Apg-2 in HCCs, and development of techniques to modulate the expression levels of these proteins will provide a novel targeted approach to control HCC cell proliferation.

Acknowledgements: The authors thank Dr. H.J. Oh for helpful suggestions, Dr. H. Itoh for the anti-Hsp110 antibody, and Dr. R.J. Mayer for reading the manuscript. This work was partly supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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