(Roche Diagnostics) by nick translation and was hybridized to metaphase chromosomes. Hybridization signals for biotin-labeled probes were detected using avidin–fluorescein (Roche Diagnostics).

3. Results

3.1. Detection of 1q21 amplicon in HCC cell lines by SNP array analysis

In the course of investigating DNA copy number aberrations in HCC cell lines by high-density SNP microarray (Affymetrix GeneChip Mapping 100K array), we found that SNU368 cells exhibited high-level amplification at 1q21 (Figs. 1A, 1B). The extent of this amplicon was estimated to be 700 kb (between Affymetrix SNP_A-1737869 and SNP_A-1662365). FISH analysis in SNU368 cells, using BAC RP11-1115K24 as a probe (Fig. 1B), revealed a strong homogeneously staining region (HSR), indicating high-level amplification (Fig. 1C). Furthermore, we determined gene dosage at the STS marker RH12271 (Fig. 1B) locus present within the amplicon by real-time quantitative PCR using DNA derived from 19 HCC cell lines. Amplification at the locus was observed in SNU368 cells (Fig. 1D). These findings confirmed amplification at 1q21 in SNU368 cells.

3.2. Amplification of 1q21 locus in primary HCC tumors

To determine whether the 1q21 region was amplified in primary tumors, we examined copy numbers at STS marker RH12271 in 36 primary HCCs by real-time quantitative PCR. Copy number changes were rated as gains if they exceeded the mean plus 2 standard deviations of the levels in normal genomic DNA derived from four peripheral blood lymphocytes. The locus was amplified in 32 of the 36 tumors (89%) (Fig. 2).

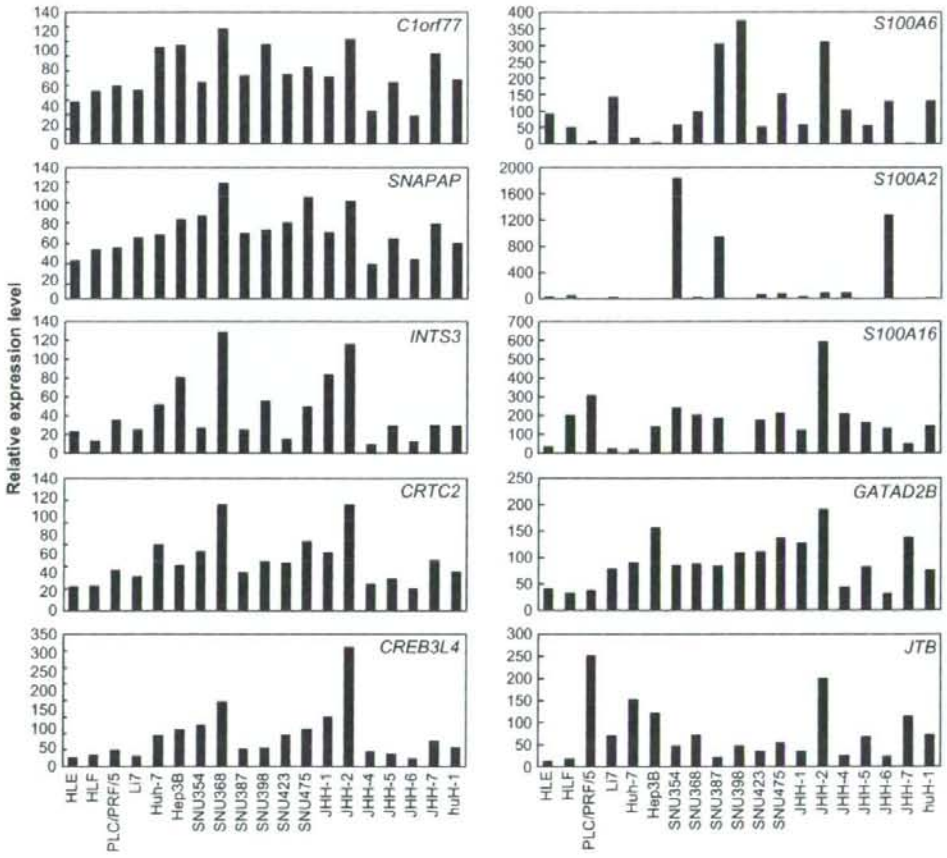

Fig. 3. Relative expression levels of 10 representative genes within the 1q21 amplicon in our panel of 19 HCC cell lines, as evaluated by real-time quantitative reverse transcriptase PCR. Results are presented as expression levels of each gene relative to a reference gene (*GAPDH*) to correct for variations in the amount of RNA. Five of the 10 genes (left: *C1orf77*, *SNAPAP*, *INTS3*, *CRTC2*, and *CREB3L4*) were overexpressed in SNU368 cells; the other 5 genes were not overexpressed (right: *S100A6*, *S100A2*, *S100A16*, *GATAD2B*, and *JTB*).

3.3. Identification of candidate target genes in 1q21 amplicon

To explore candidate target genes involved in 1q21 amplification, we determined the expression levels of all 26 genes within the amplicon in our panel of 19 HCC cell lines by real-time quantitative reverse transcriptase PCR. Five genes (*C1orf77*, *SNAPAP*, *INTS3*, *CRTC2*, and *CREB3L4*) were found to be overexpressed in SNU368 cells showing amplification at 1q21, as shown in Figure 3. In several other lines, one or more of those five genes was overexpressed without amplification. These findings suggested that the five genes are candidate targets for 1q21 amplification.

3.4. Upregulated expression of CREB3L4, INTS3, and SNAPAP in primary HCC tumors

We determined the expression levels of the five candidate genes in paired tumor and nontumor tissues from 18 HCC patients using real-time quantitative reverse transcriptase PCR. *CREB3L4*, *INTS3*, and *SNAPAP* were significantly overexpressed in 14 (78%), 14 (78%), and 13 (72%) of the tumors, respectively, compared with their counterpart nontumorous tissues (Wilcoxon signed-rank test; $P = 0.006$, $P = 0.015$, and $P = 0.014$, respectively) (Fig. 4). On the other hand, expression of *C1orf77* or *CRTC2* was not upregulated in HCC tumors. These results suggest that *CREB3L4*, *INTS3*, and *SNAPAP* are probable target for the 1q21 amplicon.

4. Discussion

The high-density SNP array analysis successfully identified high-level amplification in the narrow region at 1q21 in HCC cell lines (Figs. 1A, 1B). Frequent amplification in this region has been observed not only in HCC cell lines but also in primary HCC tumors (Figs. 1D, 2). Possible oncogenes such as *ETV3* (alias *PE-1*) [34], *MUC1* [35], and *NTRK1* (alias *TRK*) [36] are located within 1q21. Three growth-related genes located in 1q21, *HAX1*, *SHC1*, and *CKS1B*, were shown to be upregulated in HCC tumors, compared with the nontumorous tissues [37]; however, the positions of these genes were outside the amplicon we detected. The results of subsequent experiments suggest that *CREB3L4*, *INTS3*, and *SNAPAP* are probable targets for the amplicon among the 26 genes examined; the three transcripts were overexpressed in SNU368 cells that exhibited amplification (Fig. 3) and were significantly upregulated in primary HCC tumors, compared with their nontumorous counterparts (Fig. 4).

CREB3L4 (cyclic AMP responsive element binding protein 3-like 4), also referred to as AIbZIP [38], belongs to the CREB/ATF family of transcriptional factors. In humans, CREB3L4 transcripts are detected exclusively in the prostate, as well as in prostate and breast cancer cell lines [38]. Immunostaining of prostate tumors showed that CREB3L4 protein levels were higher in cancerous prostate cells than in adjacent noncancerous cells [38]. *INTS3*

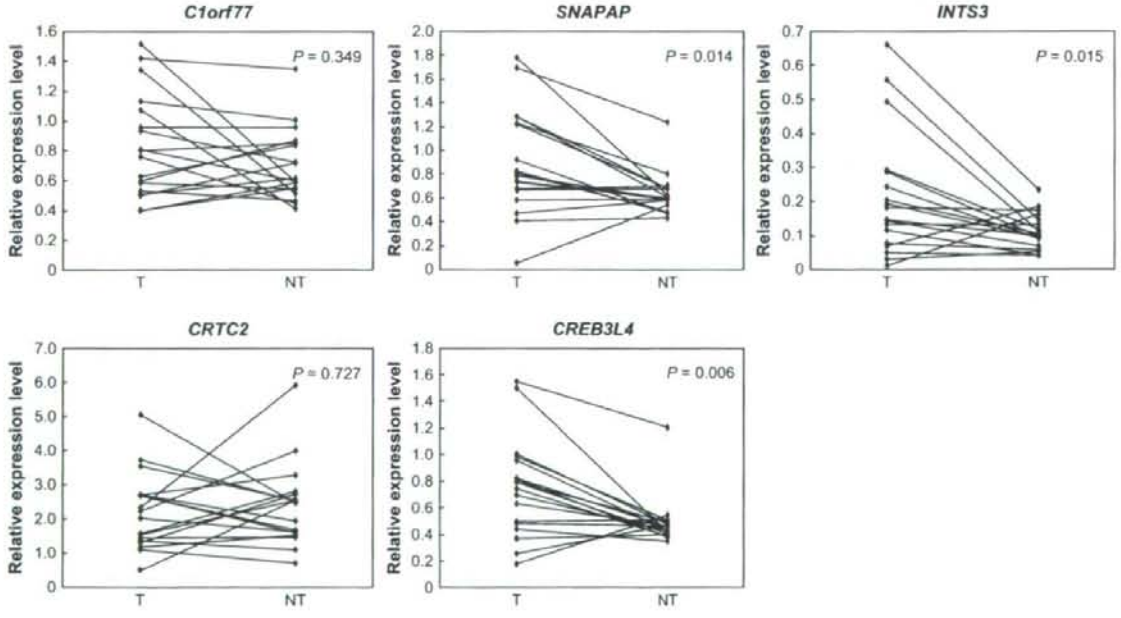

Fig. 4. Relative expression of *C1orf77*, *SNAPAP*, *INTS3*, *CRTC2*, and *CREB3L4* in paired tumor (T) and nontumor tissues (NT) from 18 patients with primary HCC. *CREB3L4*, *INTS3*, and *SNAPAP* were significantly overexpressed in primary HCC tumors. Expression levels of each gene were evaluated by real-time quantitative reverse transcriptase PCR and normalized against levels of *GAPDH*.

(integrator complex subunit 3) encodes one of the subunits of the Integrator complex. Baillat et al. [39] described an RNA polymerase II complex that contains at least 12 novel subunits, termed the Integrator, in addition to core RNA polymerase II subunits. The Integrator is found to be associated with the C-terminal domain of RNA polymerase II and mediates the processing of small nuclear RNAs (snRNAs). SNAPAP (snare-associated protein; also known as Snapin), is a component of the SNARE complex of proteins that is implicated in synaptic vesicle docking and fusion [40], and is also a component of biogenesis of lysosome-related organelles complex-1 (BLOC-1), a ubiquitously expressed multisubunit protein complex required for the normal biogenesis of specialized organelles of the endosomal-lysosomal system [41]. Little is known about the possible relationships between *INTS3* or *SNAPAP* and tumorigenesis.

Functional studies are needed to clarify the roles of these three genes in 1q21 amplification, because it is possible that coactivation of these genes leads to development and progression not only of HCCs but also of other types of tumors.

Acknowledgments

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Case Report

Non-prescription supplement-induced hepatitis with hyperferritinemia and mutation (H63D) in the *HFE* gene

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A 55-year-old Japanese woman was hospitalized because liver function tests showed an abnormality. Transaminases and biliary enzymes were markedly elevated with hyperferritinemia. Her imaging tests revealed no significant abnormality. She had been taking various non-prescription supplements for over approximately 6 months. After the supplements were discontinued her liver function gradually improved. This clinical course was suggestive of supplement-induced hepatitis. She had no history of taking supplements containing iron, so it was interesting that she had hyperferritinemia. We examined C282Y and H63D, which are important mutations in the iron-metabolizing gene, *HFE*. She was found to be heterozygous for the H63D mutation. The interaction between hyperferritinemia and supplements is unknown, but it can be speculated that some interaction between iron overload and supplements may have underlain the pathogenesis of her liver injury.

Key words: H63D mutation, heterozygous, hyperferritinemia, supplement-induced hepatitis

INTRODUCTION

IN JAPAN, MANY people take supplements for health, and many reports have identified an association between hepatotoxicity and these supplements. For example, Chinese herbal dietary supplements have been found to contain hepatotoxic agents.[1] Many different mechanisms lead to hepatotoxicity. Possibly, one of the most important susceptible factors for hepatotoxicity is genetic variability. Here, we report a case demonstrating non-prescription supplement-induced hepatitis. It was interesting that the individual had hyperferritinemia because she did not have a history of taking any iron-containing supplements. We speculate that in her case some interaction may have existed between excessive iron and supplements.

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CASE REPORT

A 55-YEAR-OLD Japanese woman was referred to Aiseikai Yamashina Hospital because tests showed abnormal liver function. In October 2004, she developed cataract. At that time, her blood test was normal. In June 2005, by chance, she underwent a blood test at the clinic of a physician and was informed of the abnormality in her liver function. On 6 July 2005, she visited another hospital. She underwent another blood test, ultrasonography (US), computed tomography (CT), and upper gastrointestinal endoscopy, all of which showed no abnormality. However, her liver function deteriorated. She was referred to our hospital on 26 July 2005 for further examination. She had no family history of liver disease and she had no history of blood transfusion, alcohol abuse, or i.v. drug use. Her height was 152 cm and her weight was 43.3 kg. Her physical findings were normal.

The laboratory data from 6 and 26 July are presented in Table 1 and Table 2. The levels of both transaminases and biliary enzymes were markedly elevated. Serological tests for hepatitis viruses were all negative. Antibodies

against cytomegalovirus (CMV) and Epstein–Barr (EB) virus showed a pattern of past infection. Autoantibodies were negative. Serum concentration of copper and ceruloplasmin were normal. Serum levels of iron and ferritin were markedly elevated. US, dynamic CT, magnetic resonance imaging (MRI) of the liver and magnetic resonance cholangiopancreatography (MRCP) revealed no significant abnormality.

At first, she denied habitual use of any types of drug. However, information from her family revealed that she had been taking various non-prescription supplements purchased from local health food stores or Internet stores after developing cataract. She had been taking soybean extract, glucosamine nutritional supplement and lutein extracted from marigoldon for over approximately 6 months before admission. All these supplements contained no iron. Because these non-prescription supplements might have caused the abnormal liver function test, all were discontinued after the patient's admission to our hospital. Her liver function gradually improved and serum transaminases returned to normal in approximately 4 weeks without any medication (Fig. 1).

Table 1 General laboratory findings for the patient taken at another hospital and at our hospital

	6 July	26 July	Normal
Peripheral blood			
White blood cell (/μL)	3780	3370	(3900–9300)
Eosinophils	ND	3%	
Neutrophils	ND	33%	
Lymphocytes	ND	49%	
Monocytes	ND	13%	
Red blood cells ($\times 10^4$/μL)	409	413	(425–571)
Platelets	24.1	21.8	(12.7–35.6)
Blood chemistry			
PT	100%	78%	
Albumin (g/μL)	4.2	4.3	(4.0–5.0)
T. Bil. (mg/dL)	0.8	1.3	(0.3–1.2)
D. Bil. (mg/dL)	ND	0.4	(0.5>)
AST (IU/L)	466	533	(33>)
ALT (IU/L)	590	693	(35>)
LDH (IU/L)	357	388	(260–485)
ALP (IU/L)	318	313	(115–360)
γ-GTP (IU/L)	147	182	(47>)
CRP (mg/dL)	0.05	0.03	(0.6>)

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; D. Bil., direct bilirubin; γ-GTP, γ-glutamyltransferase; LDH, lactate aminotransferase; ND, not determined; PT, prothrombin time; T. Bil., total bilirubin.

Table 2 Special laboratory findings on admission to our hospital

	26 July	Normal
Virus markers		
HAV IgM	(–)	(–)
HBsAg	(–)	(–)
IgM HBc Ab	(–)	(–)
HCV Ab	(–)	(–)
EBVCA IgM	(–)	(–)
EBVCA IgG	(320)	(10>)
EBNA	(40)	(10>)
CMV IgM	(–)	(–)
Immunology		
IgA (mg/dL)	200	(110–410)
IgG (mg/dL)	1784	(870–1700)
IgM (mg/dL)	165	(35–220)
ANA	40	40>
Anti DNAantibody (IU/mL)	2.7	(6.0>)
AMA	(–)	(–)
Rheumatoid factor (IU/mL)	2	(10>)
Hyaluronic acid (ng/mL)	193	(50>)
Type IV collagen 7S (ng/mL)	6.3	(5>)
Serum iron (μg/dL)	198	(48–170)
TIBC (μg/dL)	354	(235–432)
UIBC (μg/dL)	156	(108–316)
Serum ferritin (ng/mL)	983	(5.3–179.7)
Serum copper (μg/dL)	99	(78–131)
Ceruloplasmin (mg/dL)	25.2	(21–37)

AMA, anti-mitochondrial antibody; ANA, antinuclear antibody; CMV, cytomegalovirus; EBNA, Epstein Barr virus nuclear antigen; EBVCA, Epstein Barr virus capsid antigen; HAV, hepatitis A; HBc Ab, hepatitis B core antibody; HBs Ag, hepatitis B surface antigen; HCV Ab, hepatitis C antibody; TIBC, total iron binding capacity; UIBC, unsaturated iron binding capacity.

On the tenth day of hospitalization, we performed a laparoscopic examination. Her liver demonstrated a whitish uneven surface, which was suggestive of chronic liver damage (Fig. 2a,b). A liver biopsy was performed with a Vim Silverman needle. The liver specimen showed marked inflammation in the portal areas and hepatic lobules. There was grade 2 siderosis in the hepatocytes and iron storage in the Kupffer cells (Fig. 2d).[2]

The clinical course was suggestive of supplement-induced hepatitis (Fig. 1). It was interesting that she revealed hyperferritinemia, because she did not have any history of taking iron-containing supplements. We examined C282Y and H63D in her DNA, which are important mutations in the iron-metabolizing gene, *HFE*. Total genomic DNA was amplified by polymerase

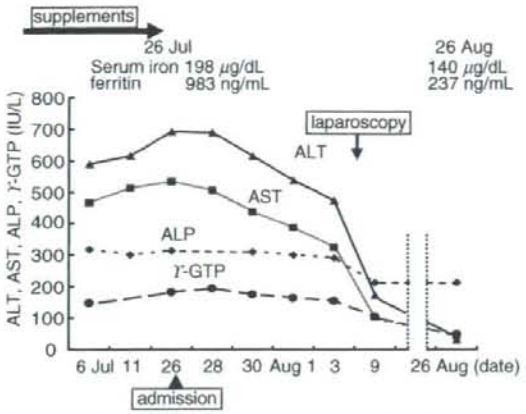

Figure 1 Clinical course of the patient. Liver function significantly improved and serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyltransferase (γ-GTP) became normal approximately 4 weeks after admission without any medication.

chain reaction (PCR) using the primers for the *HFE* gene. The PCR product was digested with *Rsa*I and *Bcl*I restriction enzymes, respectively, as previously described.[3] The results were confirmed by direct sequencing. She was found to be heterozygous for the H63D mutation (Fig. 3).

DISCUSSION

WITH THE INCREASED use of non-prescription supplements, physicians must pay attention to the potential complications of these medications. In the present case, the clinical course was highly suggestive of supplement-induced hepatitis. We used the scoring criteria presented in 'Proposal for the diagnostic criteria of drug-induced liver injury, Digestive Disease Week-Japan of 2004 (DDW-J 2004) workshop'.[4] According to the criteria, the total score was 5 and the conclusion was 'highly probable' (time to onset 1, course 2, search for non-drug causes 2). Interestingly, the patient revealed hyperferritinemia, even though she did not take any supplements containing iron. She was found to be heterozygous for the H63D mutation in the *HFE* gene.

Non-prescription supplements are categorized as food by the classification of the Ministry of Health, Labor and Welfare in Japan, and they require only an ingredients label. Some of these supplements are manufactured under poor quality control, and consist of various compounds, bases, antiseptics and trace elements. Physicians sometimes experience cases suggestive of non-prescription supplement-induced hepatitis. In many cases, it is difficult to establish a definitive causal relationship between these supplements and hepatotoxicity because of the multiplicity of ingredients and coingestants.

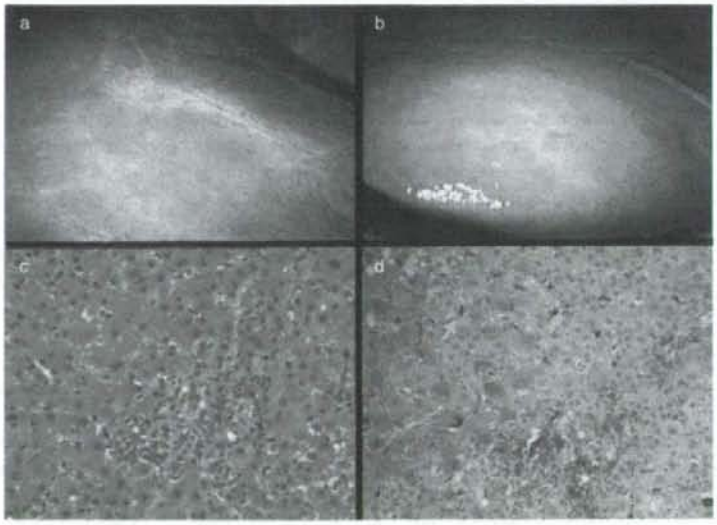

Figure 2 (a,b) Laparoscopic findings demonstrated a normal-sized liver with a whitish uneven surface. (c) Hematoxylin-eosin staining of the biopsied liver tissues showed moderate inflammation. (d) Accumulation of iron in hepatocytes was seen along with an increased number of iron-loaded Kupffer cells. Yellow arrows show iron-loaded Kupffer cells and the green arrowheads show hepatocyte siderosis.

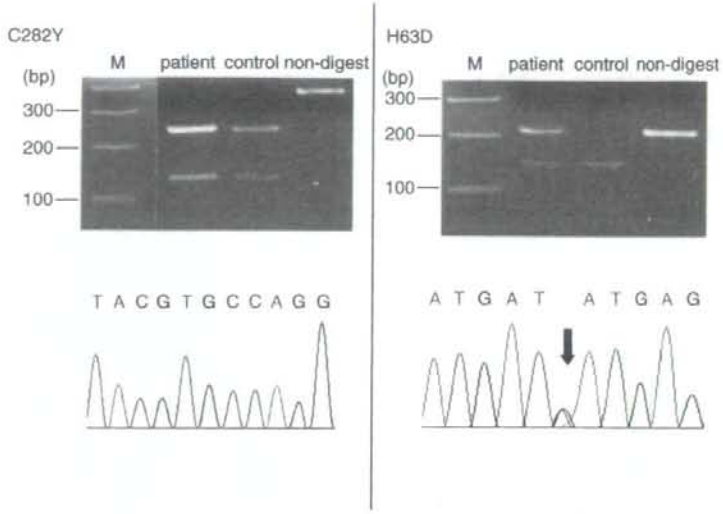

Figure 3 Determination of C282Y mutation (left panel) and H63D mutation (right panel) by restriction enzyme and direct sequencing methods. The patient was free from C282Y mutation, but was heterozygous for H63D mutation. The control was a healthy volunteer. Markers were run in lane M. The sizes of the markers are shown in base pairs (bp). Direct sequencing revealed no C282Y mutation, but demonstrated heterozygosity for H63D (G and C, black arrow).

In the present case, because the patient had been taking several types of non-prescription supplement, some of which are no longer sold, we unfortunately could not perform lymphocyte stimulation tests with and without them. However, the clinical course strongly suggested that these supplements were the inciting agents for liver injury. Additionally, it was interesting to note that she showed hyperferritinemia, although she was not taking any iron-containing supplements. Although some supplements may involuntarily contain iron, her iron overload may be attributed to the hereditary factor that she was heterozygous for the H63D mutation in the *HFE* gene.

As a representative disease of iron overload, hereditary hemochromatosis (HH) is well known. It is characterized by enhanced gastrointestinal absorption of iron to excessive accumulation in various tissues, such as liver and pancreas. C282Y missense mutation in the *HFE* gene was found to be strongly related to the occurrence of HH.[5] A second missense mutation in the *HFE* gene, H63D, was found in approximately 4% of patients with HH, but its role in iron overload is still debated.[5–8] Worldwide allele frequencies are 1.9% for C282Y and 8.1% for H63D.[9] In Japan, the C282Y mutation was not found and the H63D mutation was low in frequency at only 0.99% compared with data from Europeans.[10]

Recently, Bonkovsky *et al.* reported that patients with any *HFE* mutation had significantly higher hepatic iron scores than those without *HFE* mutations, and most parameters of serum and hepatic iron stores strongly correlated with each other.[11] In the present case, histological findings of the liver tissue revealed marked inflammation in the portal areas and hepatic lobules. In addition, there was distinct siderosis in the hepatocytes and Kupffer cells. Because iron accumulation is reported to activate Kupffer cells,[12] it can be speculated that some interaction between iron-activated Kupffer cells and supplements may have occurred *in vivo* and induced severe liver injury in this case.

Furthermore, the HFE protein is a non-classical major histocompatibility complex (MHC) class I molecule, which is known to have a variety of immunological functions apart from antigen presentation.[13,14] The etiology of drug-induced liver injury is mainly classified as toxic or immunoallergic.[15] Thus, it is possible that HFE has an immunological function distinct from iron regulation and that this immunological function may have contributed to the pathogenesis of liver injury.

In summary, although the interaction between iron overload and supplements is unknown, it can be speculated that some interaction between iron overload and supplements may have underlain the liver injury. We suggest that physicians not miss a history of taking supplements when they see patients with liver injury, especially those with hyperferritinemia.

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Association of Gankyrin Protein Expression with Early Clinical Stages and Insulin-Like Growth Factor-Binding Protein 5 Expression in Human Hepatocellular Carcinoma

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Gankyrin (also known as PSMD10) is a liver oncoprotein that interacts with multiple proteins including MDM2 and accelerates degradation of the tumor suppressors p53 and Rb. We produced a monoclonal anti-gankyrin antibody and immunohistochemically assessed the clinicopathological significance of gankyrin overexpression in 43 specimens of human hepatocellular carcinoma (HCC). Specific cytoplasmic staining for gankyrin was observed in 62.8% (27/43) of HCCs, which was significantly associated with low TNM stage ($P = 0.004$), no capsular invasion ($P = 0.018$), no portal venous invasion ($P = 0.008$), and no intrahepatic metastasis ($P = 0.012$). The cumulative survival rate of patients with gankyrin-positive HCC was significantly higher than that with gankyrin-negative HCC ($P = 0.037$). p53 and MDM2 were positively stained by antibodies in 30.2% and 23.3%, respectively, of HCCs, but neither was inversely associated with gankyrin expression. In the Huh-7 human HCC cell line, overexpression of gankyrin up-regulated expression of insulin-like growth factor binding protein 5 (IGFBP-5), whereas suppression of gankyrin expression by siRNA down-regulated it. Supression of IGFBP-5 expression inhibited proliferation of Huh-7 cells as well as U-2 OS osteosarcoma cells. In HCC specimens, positive staining for IGFBP-5 was observed by immunohistochemistry in 41.9% (18/43), and the level of expression was significantly correlated with that of gankyrin ($rho = 0.629$, $P < 0.001$). *Conclusion:* These results suggest that gankyrin plays an oncogenic role(s) mainly at the early stages of human hepatocarcinogenesis, and that IGFBP-5 inducible by gankyrin overexpression may be involved in it. (HEPATOLOGY 2008;47:493-502.)

Liver cancer is the sixth most common cancer worldwide (626,000 or 5.7% of new cancer cases) and the third most common cause of death from cancer (598,000) in 2002.[1] Eighty-two percent of cases are in developing countries, and the areas of high incidence are sub-Saharan Africa, eastern and southeastern Asia, and Melanesia. Histologically, more than 90% of the primary liver cancers are hepatocellular carcinomas (HCCs). Although there are several modalities of treatment for HCC, most patients present with unresectable tumors, and nonsurgical treatments are minimally effective at best.[2,3] Even for those patients who undergo surgical resection, the recurrence rate is very high and the prognosis is poor.[2,4-6] It is therefore important to clarify the mechanisms of human hepatocarcinogenesis and identify molecular targets to develop novel diagnostic, therapeutic, and preventive strategies.

By constructing subtracted complementary DNA (cDNA) libraries, we have previously identified 19 genes overexpressed in HCCs including 2 novel genes.[7,8] One of them was named gankyrin (gann-ankyrin repeat pro-

Abbreviations: 3A6C2, mouse monoclonal anti-gankyrin antibody; cDNA, complementary DNA; HCC, hepatocellular carcinoma; IGF, insulinlike growth factor; IGFBP-5, insulin-like growth factor-binding protein 5; MDM2, mouse double minute 2; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; siRNA, short interfering RNA; TNM, tumor-node-metastasis.

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tein; "gann" in Japanese means cancer).[9] Gankyrin (also called PSMD10) consists of 7 ankyrin repeats, and its messenger RNA (mRNA) was overexpressed in 34 of 34 HCCs analyzed.[9,10] Independently, gankyrin was isolated as the p28 component or the interactor of the S6b subunit of the 19S regulator of the 26S proteasome.[11,12] The ankyrin repeat is the functional domain involved in protein–protein interactions, and gankyrin has been shown to interact with multiple proteins in addition to S6b. Gankyrin binds to retinoblastoma protein (Rb) and cyclin-dependent kinase (Cdk4), and accelerates phosphorylation and degradation of Rb, which results in release of the E2F transcription factor to activate DNA synthesis genes.[9,13] Gankyrin seems to play a role in cell cycle progression in noncancerous cells as well. Overexpression of gankyrin shortens population doubling time of NIH/3T3 mouse fibroblasts,[9] and its up-regulation correlates with cell cycle progression in normal rat primary hepatocytes, oval cells, and human hepatocytes.[14,15]

Overexpression of gankyrin confers tumorigenicity to NIH/3T3 cells and inhibits apoptosis in cultured human tumor cells exposed to chemotherapeutic agents.[10] The anti-apoptotic activity is attributable, at least partly, to increased degradation of p53, resulting in the reduced transcription of the p53-dependent proapoptotic genes.[16] Gankyrin binds to the E3 ubiquitin ligase MDM2 *in vitro* and *in vivo*, which increases p53–MDM2 association, thereby facilitating the ubiquitination and subsequent proteasomal degradation of p53 by MDM2. Gankyrin also controls MDM2 auto-ubiquitination and degradation, especially in the absence of p53.[16]

We produced a mouse monoclonal antibody against human gankyrin and assessed the expression of gankyrin protein in surgically resected HCC specimens by immunohistochemistry. Correlation of gankyrin positivity with clinicopathological findings and expression of p53 and MDM2 in HCC was analyzed. Furthermore, we demonstrated that expression of insulin-like growth factor-binding protein 5 (IGFBP-5) is inducible by overexpression of gankyrin in HCCs.

Patients and Methods

Patients and Specimens. HCC tissues and their corresponding noncancerous liver tissues were obtained from 43 and 32 patients, respectively, who had undergone curative hepatectomy at the University Hospital of Kyoto Prefectural University of Medicine between 1992 and 2000. The specimens used were routinely processed, formalin-fixed, and paraffin-embedded. After hematoxylin-eosin staining, all samples were diagnosed as HCC and the tumor-node-metastasis (TNM) classification was made according to the fourth edition of the general rules for the clinical and pathological study of primary liver cancer proposed by the Liver Cancer Study Group of Japan.[17] The demographic profiles of the patients are summarized in Table 1. For western blot analysis, HCCs and noncancerous liver tissues were obtained from 3 patients undergoing liver transplantation at the University Hospital of Kyoto Prefectural University of Medicine between 2004 and 2006. No donor organs were obtained from executed prisoners or other institutionalized persons. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review boards. Written informed consents were obtained from all patients for subsequent use of their resected tissues.

Table 1. Patient and Tumor Characteristics

Characteristic	Number (Percentage)
Number of patients	43
Sex distribution	
Male	27 (62.8%)
Female	16 (37.2%)
Age (years)	25-78, median 65
Virus marker	
HBV(+)/HCV(−)	6 (14.0%)
HBV(−)/HCV(+)	28 (65.0%)
HBV(+)/HCV(+)	3 (7.0%)
HBV(−)/HCV(−)	6 (14.0%)
AFP(ng/mL)	3.5-39999, median 90
Tumor size (cm)	1.6-17.0, median 4.0
Liver cirrhosis	
Yes	29 (67.5%)
No	14 (32.5%)
Chronic hepatitis	13 (30.2%)
Normal	1 (2.3%)
TNM stage	
I	4 (9.3%)
II	22 (51.1%)
III	8 (18.6%)
IV	9 (21.0%)
Histological differentiation	
Well	12 (27.9%)
Moderate	25 (58.1%)
Poor	6 (14.0%)
Capsular formation	
Yes	36 (83.7%)
No	7 (16.3%)
Capsular invasion	
Yes	14 (32.6%)
No	29 (67.4%)
Portal venous invasion	
Yes	9 (20.9%)
No	34 (79.1%)
Intrahepatic metastasis	
Yes	16 (37.2%)
No	27 (62.8%)

Abbreviations: HCV(+), anti-hepatitis C virus antibody positive; HBV(+), hepatitis B surface antigen positive; (−), negative; AFP, serum alpha-fetoprotein.

Cell Culture and Transfection. Huh-7 human HCC cells, U-2 OS human osteosarcoma cells, 293T human embryonic kidney cells, mouse lymph node cells, and P3X63Ag8U.1 mouse myeloma cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies, NY) supplemented with 10% fetal bovine serum as described.[16] To assess viable cell numbers, we used the Dojindo Cell Counting Kit-8 (CCK8 kit, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

The 293T, Huh-7, and U-2 OS cells were transfected with plasmid DNA by using the calcium phosphate method or FuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) as described.[16] Short interfering RNA (siRNA) were transfected at a final concentration of 25 nM by using siPORT NeoFX Transfection Agent (Ambion, Austin, TX) following the manufacturer's instructions. Twenty-four hours after transfection, the medium was replaced with fresh medium containing fetal bovine serum, and the culture was continued for another 24 or 48 hours. Then, the cells were harvested for analysis. All transfection assays were repeated at least 3 times.

Plasmids and siRNA. Human wild-type gankyrin cDNAs, full coding sequence and deletion mutants, were cloned into the mammalian expression vector pMKIT-NEO and expressed as hemagglutinin (HA)-tagged proteins (Fig. 1A). Full-length gankyrin was expressed without a tag as well. To obtain recombinant human gankyrin protein, the full-length cDNA was cloned into an expression vector derived from pET28 (Novagen, EMD Biosciences Inc., San Diego, CA) and expressed as hexahistidine-tagged protein.

To down-regulate gene expression, Silencer Predesigned siRNAs for gankyrin (Ambion) and Stealth Select iRNA: for IGFBP-5 (Invitrogen, Tokyo, Japan), were used together with respective control RNAs.

Antibodies. To obtain monoclonal antibodies against human gankyrin, recombinant (His)6-gankyrin protein was used as an immunogen. It was dissolved in phosphate-buffered saline (1 mg/mL) and emulsified with an equal volume of Freund's complete adjuvant (Difco, Becton-Dickinson, Franklin Lakes, NJ). Two female BALB/c mice were injected with the emulsion (50 μL/mouse) in the footpad. Two weeks after immunization, the inguinal lymph node cells (4×10^7 cells) were fused with P3X63Ag8U.1 myeloma cells (1×10^7) using polyethylene glycol 1500 (Roche Diagnostics). Fused cells were cultured in 96-well plates at 2×10^5 cell/well. The supernatants were assayed for the anti-gankyrin antibody titer by an enzyme-linked immunosorbent assay using recombinant His-tagged, glutathione-S-transferase (GST)-tagged, and nontagged gankyrin proteins. Selected relevant hybridomas were cloned by the limiting dilution method, and the isotypes of secreted monoclonal antibodies were determined by the IsoStrip kit (Roche Diagnostics) following the manufacturer's instructions. Finally, an IgG2b kappa monoclonal antibody that showed the highest affinity for gankyrin was obtained and named 3A6C2.

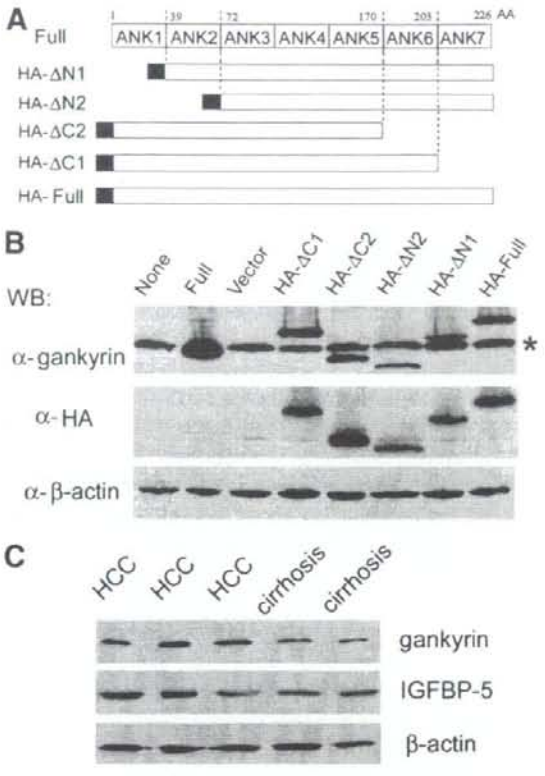

Fig. 1. Recognition of gankyrin protein by the monoclonal antibody. (A) Structures of wild-type gankyrin (Full) and its deletion mutants. Numbers on top, N- and C-terminal amino-acid residues. ANK, ankyrin repeat. Black bars, HA tags. (B) Specificity of the antibody. 293T cells were transfected with plasmids expressing the indicated proteins. Cell lysates were analyzed by western blotting (WB) using the anti-gankyrin monoclonal antibody (3A6C2), anti-HA antibody, and anti-β-actin antibody. *Mobility of the endogenous gankyrin. Representative results of 3 repeated experiments are shown. (C) Detection of gankyrin protein in tissues. Lysates were made from hepatocellular carcinoma (HCC, n = 3) and cirrhotic liver tissues (n = 2), and analyzed by WB using antibodies for indicated proteins. HA, hemagglutinin.

For western blot analysis, mouse monoclonal anti-gankyrin antibody (3A6C2), goat polyclonal anti–IGFBP-5 antibody (R&D Systems Inc., Minneapolis, MN), mouse monoclonal anti-HA antibody (12CA5, Roche Diagnostics), and mouse monoclonal anti–β-actin antibody (Chemicon International, Temecula, CA) were

used. Horseradish peroxidase–conjugated secondary antibodies against mouse or goat immunoglobulins were obtained from DAKO (Kyoto, Japan).

For immunohistochemistry, mouse monoclonal anti-gankyrin (3A6C2), anti-MDM2 (Ab-4, Oncogene research products, Boston, MA), and anti-p53 (DO-7, DAKO) antibodies, rabbit polyclonal anti-IGFBP-5 antibody (GroPep, Thebarton, Australia), and horseradish peroxidase–conjugated secondary antibodies against mouse or rabbit immunoglobulins (DAKO) were used.

Analysis of Gene Expression. Extraction of RNA, preparation of cell and tissue lysates, and western blot analysis were performed as described.[9] Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using ABI PRISM 7900 (Applied Biosystems, Foster City, CA) and a 1-step QuantiTect RT-PCR Kit (Qiagen, Cowley, UK) according to the manufacturer's instructions. PCR conditions were 50°C for 30 minutes and 95°C for 15 minutes, followed by 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. Specific PCR amplification products were detected by SYBR Green. Transcripts of β-actin were quantified as control. Primer sequences used were as follows: IGFBP-5, AAGAAGCTGACCCAGTCCAA and GAATCCTTTGCGGTCACAAT; gankyrin, GCAACTTGGAGTGCCAGTGAA and TCACTTGAGCACCTTTTCCCA; β-actin, CTACGTCGCCCTGGACTTCGAGC and GATGGAGCCGCCGATCCACACGG.

The immunohistochemical staining was performed on 4-μm-thick paraffin sections of tissues fixed in buffered formalin. The sections were pretreated with 10 mM citrate buffer (pH 6.1) in a microwave oven for 5 minutes. Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 for 10 minutes. The sections were incubated with 10% fetal bovine serum for 30 minutes to reduce nonspecific binding, followed by incubation with the primary antibody at 4°C overnight. They were subsequently incubated with horseradish peroxidase–conjugated anti-mouse or rabbit immunoglobulin antibody for 30 minutes. The enzymatic reaction was developed in a freshly prepared solution of 3,3′-diaminobenzidine tetrahydrochloride using DAKO Liquid DAB Substrate-Chromogen Solution for 10 minutes at room temperature. The sections were then counterstained with hematoxylin. The staining pattern, the distribution of the immunostaining in each tissue, and the intensity of the staining were studied in detail. Negative controls were conducted by substituting normal sera of each animal for the primary antibodies. When immunoreactivities were heterogenously observed, cases with moderate or strong staining of nucleus or cytoplasm in more than 5% of the cells were considered positive. To analyze the correlation of the expression levels of gankyrin and IGFBP-5, the staining intensity was expressed as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive). In each case the immunoreactivity was determined in 5 random high-powered fields and the count was done independently by 2 observers.

Statistical Analysis. Categorical variables were compared using Fisher's exact test. Paired comparison of continuous data was performed using the Wilcoxon signed ranks test. To assess whether the 2 variables covary, Spearman's rank correlation coefficient was determined. Cumulative survival curves were calculated by the Kaplan-Meier method and analyzed by the log-rank test. All statistical analyses were performed using the JMP statistical software package (SAS Institute Inc., Cary, NC). A P value less than 0.05 was considered statistically significant.

Results

Clinicopathological Profiles. Forty-three patients with HCC were recruited in this study, including 27 men and 16 women, with ages ranging from 25 to 78 (median 65) years old. Clinicopathological profiles of the patients and their HCCs are shown in Table 1. Antibody to hepatitis C virus was found in sera of 72% of the patients, and hepatitis B virus surface antigen was positive in 21%.

According to the TNM staging, 60% were stage I to II and 40% were stage III to IV. In noncancerous portions of the resected livers, cirrhosis and chronic hepatitis[18] were found in 68% and 30%, respectively, of the specimens, whereas only 1 (2%) was of normal histology. Fibrocapsular formation surrounding HCC was observed in 84% and capsular invasion by HCC cells in 33%. Portal vein involvement and satellite nodules suggesting intrahepatic metastasis were found in 21% and 37%, respectively.

Detection of Gankyrin with the Monoclonal Anti-gankyrin Antibody. To determine the specificity of the monoclonal anti-gankyrin antibody 3A6C2, we expressed wild-type full-length or truncated gankyrin (Fig. 1A) in 293T cells. The antibody detected all mutants of gankyrin, suggesting that the epitope exists within the third and fifth ankyrin-repeat region (Fig. 1B). The antibody recognized the endogenous gankyrin as well, and no major cross-reacting band was observed.

Because gankyrin mRNA is known to be overexpressed in most HCCs,[9] we analyzed the levels of gankyrin protein in HCCs and surrounding noncancerous liver tissues using the 3A6C2 antibody. The protein level of gankyrin was higher in HCC tissues than in noncancerous tissues

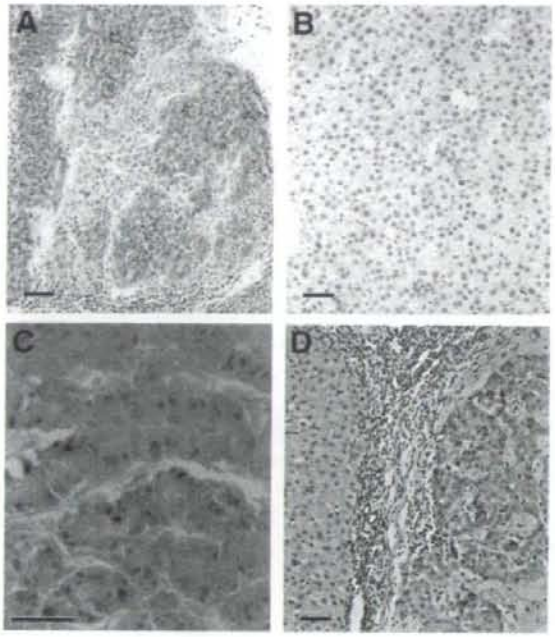

Fig. 2. Immunohistochemical detection of gankyrin in hepatocellular carcinoma (HCC). HCC sections were stained with mouse monoclonal anti-gankyrin antibody, and counterstained with hematoxylin. Positive immunostaining appears brown. (A) Positive staining for gankyrin in the cytoplasm of most HCC cells. (B) Barely detectable gankyrin signal in some HCC cells. (C) Presence of gankyrin in the nucleus of some HCC cells. (D) Stronger staining for gankyrin in HCC cells (right) than the neighboring cirrhotic hepatocytes (left). Bar, 50 μm.

(Fig. 1C). The mobilities of the gankyrin band were not different among samples.

Immunohistochemical Analysis of Gankyrin Expression. We next examined the expression of gankyrin protein in HCC and noncancerous liver tissues by immunohistochemistry. The gankyrin signal was observed mainly in the cytoplasm and occasionally in the nucleus of HCC cells (Fig. 2A-C). Although at lower levels compared with those in HCCs, weak but reproducible gankyrin signals were observed in the cytoplasm of the hepatocytes in the noncancerous tissues (Fig. 2D). Expression of gankyrin was not detected in the bile duct cells, blood endothelial cells, or other nonparenchymal cells in the liver tissues. Of 43 HCCs examined, the cytoplasm was stained positively for gankyrin in 27 (63%), and 9 of them (21%) were also positive for nuclear staining. Of 32 noncancerous liver tissues available, gankyrin was positive in 17 (53%).

As shown in Table 2, we analyzed an association between gankyrin protein expression and clinicopathological findings. No significant association between gankyrin expression in HCC cells and sex, age, tumor size, fibrotic change in noncancerous liver tissues, differentiation of the tumor cells, or hepatitis B or C virus infection was observed. Positive cytoplasmic staining for gankyrin of HCC cells was significantly associated with low TNM stage (stage I or II; $P = 0.004$), no capsular invasion ($P = 0.018$), no portal venous invasion ($P = 0.008$), and no intrahepatic metastasis ($P = 0.012$) of HCC. In noncancerous liver tissues, positive gankyrin staining of hepatocytes was associated with the cytoplasmic gankyrin positivity of HCC cells of the same patient ($P = 0.021$, Table 3), but not with the parameters examined except for the serum alpha-fetoprotein level ($P = 0.015$, Table 2).

Because expression of gankyrin affects the degradation of p53 and MDM2,[16] we examined the expression of p53 and MDM2 as well as gankyrin in HCCs. By immunohistochemistry, nuclear expression of p53 and MDM2 were detected in 30% and 23%, respectively, of 43 HCCs (Fig. 3, Table 3). Positive staining for gankyrin was not associated with the staining for p53 nor MDM2 in HCC cells.

Up-regulation of IGFBP-5 Expression by Gankyrin in HCCs. Preliminary microarray analysis of the cDNA libraries prepared from U-2 OS cells and Huh-7 cells overexpressing gankyrin suggested that IGFBP-5 mRNA was up-regulated by gankyrin (A. Umemura and J. Fujita, unpublished data). Real-time RT-PCR analysis con-

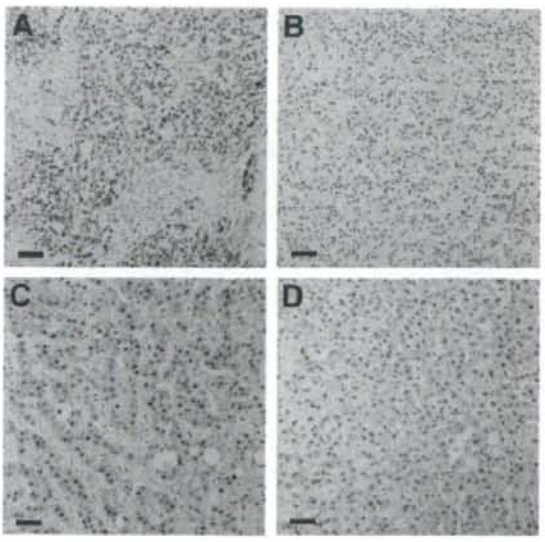

Fig. 3. Immunohistochemical detection of p53 and MDM2 in hepatocellular carcinoma (HCC). HCC sections were stained with antibodies specific to p53 (A and B) or MDM2 (C and D), and counterstained with hematoxylin. Positive immunostaining appears brown. (A) Positive staining for p53 in the nucleus of most HCC cells. (B) Negative p53 in HCC cells. (C) Positive staining for MDM2 in the nucleus of most HCC cells. (D) Negative MDM2 in HCC cells. Bar, 50 μm.

Table 2. Gankyrin Expression and Clinicopathological Characteristics

	Gankyrin Expression in the Cytoplasm of					
	HCC			Noncancerous Liver		
	Negative (n = 16)	Positive (n = 27)	P value	Negative (n = 15)	Positive (n = 17)	P value
Sex distribution						
Male	12	15	0.328	10	11	1.000
Female	4	12		5	6	
Median age (years)	64	65	0.696	63	62	0.649
Virus marker			NS			NS
HBV(+)/HCV(−)	3	3		2	2	
HBV(−)/HCV(+)	10	18		11	11	
HBV(+)/HCV(+)	1	2		2	0	
HBV(−)/HCV(−)	2	4		0	4	
Median AFP (ng/mL)	63.0	95.0	0.890	25.0	199.0	0.015
Median tumor size (cm)	4.5	4.0	0.098	4.5	4.0	0.372
Liver cirrhosis (+)	9	20	0.316	9	13	0.450
TNM stage						
I and II	5	21	0.004	8	12	0.467
III and IV	11	6		7	5	
Histological differentiation						
Well	5	7	0.737	6	3	0.243
Moderate and poor	11	20		9	14	
Capsular formation (+)	15	21	0.229	12	13	1.000
Capsular invasion (+)	9	5	0.018	4	6	0.712
Portal venous invasion (+)	7	2	0.008	4	3	0.678
Intrahepatic metastasis (+)	10	6	0.012	6	5	0.712
Gankyrin nuclear expression						
Yes	0	9	0.016	2	5	0.403
No	16	18		13	12	

Abbreviations: HCV, anti-hepatitis C virus antibody; HBV, hepatitis B surface antigen; (+), positive or present; (−), negative or absent; AFP, serum alpha-fetoprotein; NS, not significant between any groups or combinations thereof.

firmed that overexpression of gankyrin increased the IGFBP-5 mRNA levels 5.2-fold and 1.7-fold (mean, n = 3 each) in U-2 OS and Huh-7 cells, respectively, and western blot analysis demonstrated that the protein levels were increased as well (Fig. 4A). Conversely, when gankyrin expression was suppressed by siRNA, IGFBP-5 expression was down-regulated (Fig. 4B). In 2 of 3 HCC tissues overexpressing gankyrin, the levels of IGFBP-5 protein were higher compared with those in noncancerous tissues (Fig. 1C). To identify a role that IGFBP-5 might play in HCC cells, we next suppressed IGFBP-5 expression by siRNA. No apoptosis was induced, but viable cell numbers were decreased in Huh-7 as well as U-2 OS cells (Fig. 4C,D, and data not shown), suggesting a growth-promoting effect of IGFBP-5.

The expression of IGFBP-5 was further examined immunohistochemically in 43 HCC and 32 noncancerous liver tissues (Fig. 5, Table 3). In 42% of HCCs, IGFBP-5 was positively stained in the cytoplasm of HCC cells (Fig. 5A). IGFBP-5 was also detected, although at lower levels, in the cytoplasm of hepatocytes in 28% of the noncancerous tissues (Fig. 5B-D), but not in bile duct cells, blood endothelial cells, or other nonparenchymal cells.

Specific cytoplasmic staining for IGFBP-5 in HCC cells was associated with low TNM stage (stage I or II; $P = 0.013$), no portal venous invasion ($P = 0.006$), low serum alpha-fetoprotein value ($P = 0.031$), and small tumor size ($P = 0.009$). No association with capsular invasion or intrahepatic metastasis was observed. There was a significant association between positivities for IGFBP-5 and

Table 3. Gankyrin Expression and Molecular Histological Markers

	Gankyrin Expression in HCC		
	Negative	Positive	P value
Gankyrin expression in non-HCC			
Negative (n = 15)	8	7	0.021
Positive (n = 17)	2	15	
p53 expression in HCC			
Negative (n = 30)	11	19	1.000
Positive (n = 13)	5	8	
MDM2 expression in HCC			
Negative (n = 33)	14	19	0.276
Positive (n = 10)	2	8	
IGFBP-5 expression in HCC			
Negative (n = 25)	13	12	0.026
Positive (n = 18)	3	15	
IGFBP-5 expression in non-HCC			
Negative (n = 23)	14	9	0.011
Positive (n = 9)	1	8	

Abbreviations: HCC, hepatocellular carcinoma; non-HCC, noncancerous portion of the resected liver; IGFBP-5, insulin-like growth factor-binding protein 5.

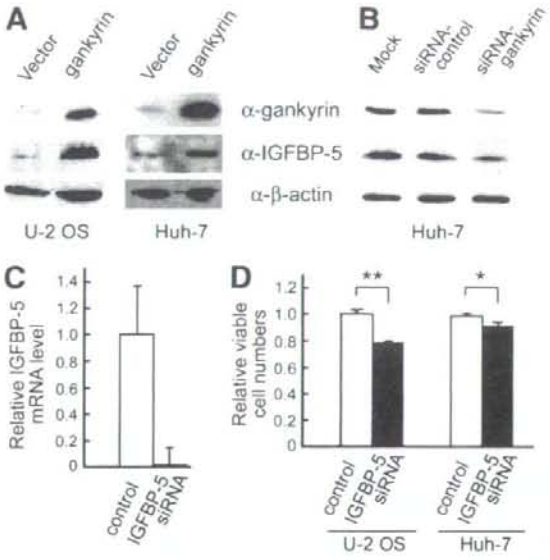

Fig. 4. Induction of IGFBP-5 by gankyrin. (A) U-2 OS cells (lanes 1 and 2) and Huh-7 cells (lanes 3 and 4) transiently transfected with plasmids expressing gankyrin or vector alone were analyzed for expression of IGFBP-5 by western blotting using the indicated antibodies. Representative results from more than 3 experiments are shown. (B) Huh-7 cells, mock transfected or transfected with siRNA for gankyrin or control RNA as indicated, were analyzed as in (A). (C) Suppression of IGFBP-5 expression by siRNA. Huh-7 cells were transfected with control RNA or IGFBP-5-specific siRNA. IGFBP-5 transcript levels were determined by real-time RT-PCR and normalized with β-actin levels. Results from 3 repeats were averaged and expressed relative to control. Error bars refer to standard deviation of the average quantitated results. (D) Effect of IGFBP-5 down-regulation on cell growth. U-2 OS and Huh-7 cells were transfected with IGFBP-5 siRNA or control RNA, and 72 hours later viable cell numbers were determined. Values are mean ± standard deviation (n = 3) and expressed relative to controls. ** and *, $P < 0.01$ and $P < 0.05$, respectively.

gankyrin (Table 3), and the levels of expression covaried both in HCCs ($\rho = 0.629$, $P < 0.001$) (Fig. 5E) and noncancerous hepatocytes ($\rho = 0.606$, $P < 0.001$) (Fig. 5F).

Expression of Gankyrin in HCC and Patient Prognosis. When we examined the relationship between gankyrin expression in HCC cells and the survival of patients after surgical resection, a significant difference was observed between the patients with gankyrin-positive HCCs and those with gankyrin-negative HCCs (Fig. 6). We found no significant difference in the survival rates between the patients whose HCCs stained positively and negatively for p53, MDM2, or IGFBP-5.

Discussion

Gankyrin is as an oncogene, mRNA of which is overexpressed in almost all human HCCs.[9,19] Although less frequent, gankyrin has been found by RNA dot blot analysis to be overexpressed in additional tumors including those of the breast, colon, rectum, stomach, small intestine, pancreas, ovary, lung, and thyroid (A. Umemura and J. Fujita, unpublished data). In the current study, we immunohistochemically examined the gankyrin protein expression in HCCs using the monoclonal anti-gankyrin antibody and found that the protein was highly expressed in the cytoplasm of 63% of HCCs. Tan et al.[20] has simi-

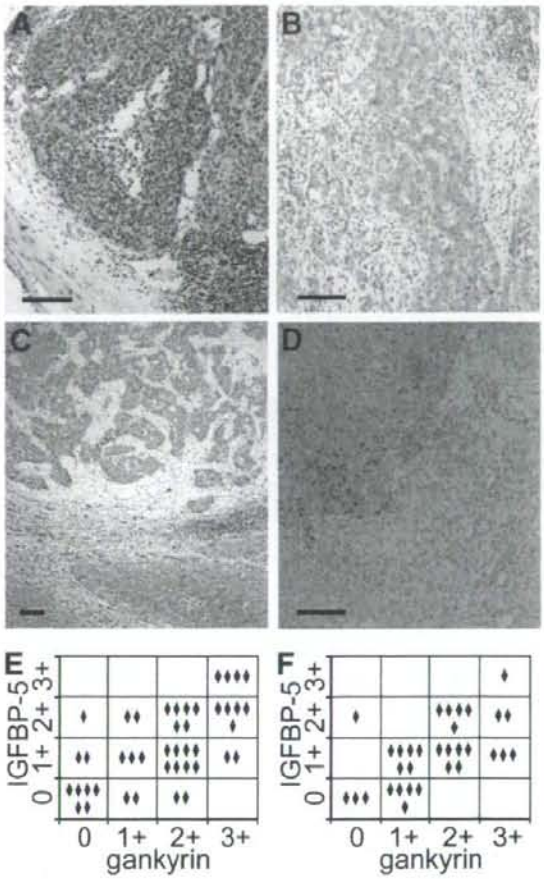

Fig. 5. Immunohistochemical detection of IGFBP-5 in hepatocellular carcinoma (HCC). HCC sections were stained with anti-IGFBP-5 antibody and counterstained with hematoxylin. Positive immunostaining appears brown. (A) Positive staining for IGFBP-5 in the cytoplasm of HCC cells, especially at the invasive boundaries. (B) Presence of IGFBP-5 in noncancerous cirrhotic hepatocytes. (C) Stronger staining for IGFBP-5 in HCC cells (upper) than the neighboring cirrhotic hepatocytes (lower). (D) Positive staining for IGFBP-5 in HCC cells (upper left), but negative in cirrhotic cells (lower right). Bar, 100 μm. (E) Correlation of expression levels of gankyrin and IGFBP-5 in HCCs. The immunostaining levels were expressed as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive). Each diamond represents 1 case. The Spearman's rho = 0.629, $P < 0.001$. (F) Correlation of expression levels of gankyrin and IGFBP-5 in noncancerous hepatocytes determined as in (E). The Spearman's rho = 0.606, $P < 0.001$.

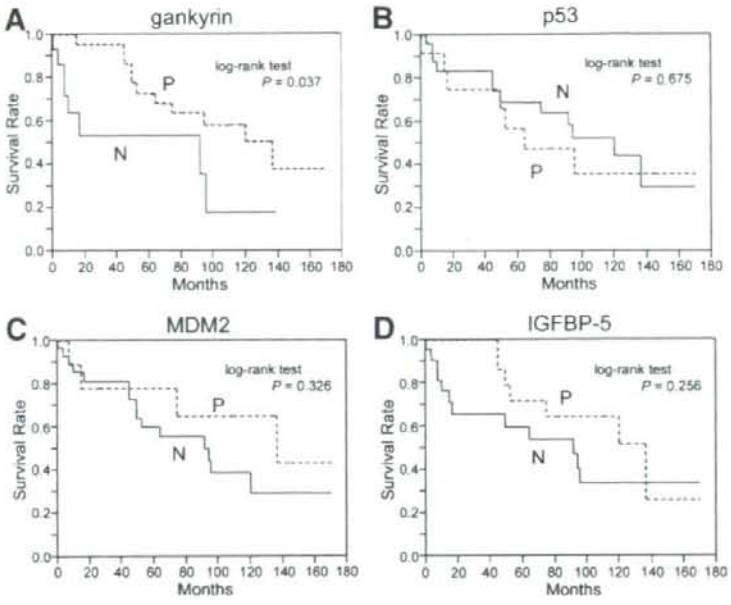

Fig. 6. Survival of patients and expression of molecular markers. The Kaplan-Meier method was used to determine the patient survival and log-rank test to compare survival between patients with HCC grouped according to (A) gankyrin positivity, (B) p53 positivity, (C) MDM2 positivity, and (D) IGFBP-5 positivity. P, positive. N, negative.

larly found overexpression of gankyrin protein in 60% of HCCs using a polyclonal antibody. The reason why the protein is not overexpressed in one-third of HCCs despite overexpression of its mRNA is unknown. The posttranscriptional, translational, and posttranslational regulations of gankyrin expression remain to be elucidated.

According to the 15th follow-up survey by the Liver Cancer Study group of Japan, the cumulative survival rates after surgical removal of HCC are 52.3% and 27.3% at 5 and 10 years, respectively, and better survival rates are associated with fewer numbers of tumors, lack of portal venous invasion, and early clinical stages.[4-6] Consistent with these observations, gankyrin positivity of HCC was associated with low TNM stage, lack of capsular invasion, portal venous invasion, and intrahepatic metastasis, and better prognosis of the patients. Patients with hyperdiploid acute lymphoblastic leukemia with more than 50 chromosomes, one of the 6 subtypes of pediatric acute lymphoblastic leukemia, have an excellent prognosis compared with other subtypes, and interestingly, overexpression of gankyrin is 1 of the diagnostic and subclassification markers for it.[21] Expression of gankyrin protein may be used as a marker for better prognosis of the patients with HCC as well.

The gankyrin oncoprotein plays a key role in regulation of cell cycle and apoptosis, at least in cultured cells, by inhibiting Rb and p53.[10] In a rodent hepatocarcinogenesis model, hypermethylation of the p16INK4A gene and p53 mutation appear at a late stage, whereas gankyrin is overexpressed from early after carcinogen treatment, preceding the loss of Rb protein and adenoma formation.[22] Clinically, p53 mutation is not so frequent in HCCs (15%-30%), especially in low-grade or low-stage HCCs.[23,24] Tan et al.[20] have immunohistochemically detected gankyrin overexpression in 82%, 63%, and 22% of Edmondson's grade I to II, III, and IV HCCs, respectively. We observed gankyrin positivity in 81% and 35% of low and high TNM stage HCCs, respectively. These results suggest that gankyrin plays an important role(s) at early stages of hepatocarcinogenesis by suppressing Rb, p53 and possibly other tumor suppressors. In advanced HCCs, by contrast, oncogenic mutations probably have accumulated in many genes including p53, and overexpression of gankyrin may not be so crucial as in early stage HCCs. This could explain the present association of gankyrin-negative HCCs with poorer prognosis and the finding that both cases of gankyrin-negative HCCs with gankyrin-positive noncancerous hepatocytes belonged to high TNM stages. This is, however, one of several possible explanations, and further work is necessary to clarify the exact reasons for the observed association.

By immunohistochemical staining, p53 has been detected in 20% to 30% of HCCs.[25, 26] Although strong immunohistochemical reactivity for p53 may not be an indicator of the presence of p53 gene mutations as initially suggested,[26] it has been associated in some studies with higher proliferative activity, lower differentiation of HCC cells, or poorer survival of patients. Endo et al.[27] immunohistochemically detected MDM2 in 28 of 107 (26%) HCCs, and the positive expression correlated with

the presence of p53 mutation and poorer prognosis, although it also correlated with smaller HCC size and the absence of vascular invasion. We immunohistochemically detected the expression of p53 and MDM2 in 30% and 23%, respectively, of HCCs, which is in accord with other studies, but no correlation was seen between expression and survival of the patients. Gankyrin accelerates degradation of Rb, p53, and MDM2 in cultured cells.[9,16] Although some correlation between expression of gankyrin and Rb has been suggested in HCC tissues,[20] we did not observe significant relationship between the gankyrin positivity and negative staining for p53 nor MDM2. The analysis of individual cells for protein expression, for example by double 2-color immunostaining, may have revealed the presence of some relationship. But most probably, our finding reflects complex interrelated mechanisms regulating the levels of these proteins and also suggests that the relevance of the effects of gankyrin on p53, MDM2, and Rb demonstrated in cultured cells to human hepatocarcinogenic process remains to be firmly established.

The 6 members of IGFBP family (IGFBP-1 through IGFBP-6) are important components of the insulin-like growth factor (IGF) axis, and regulate the activity of both IGF-I and IGF-II polypeptide growth factors.[28] IGF-I, IGF-II, and their receptors are expressed in a wide variety of cells, and the liver is the main source of circulating IGF-I. IGFBPs are also secreted by many cell types, and their expression is regulated in a cell-dependent and tissue-type–dependent manner. In the current study, we found up-regulation of IGFBP-5 mRNA and protein levels by overexpression of gankyrin in human osteosarcoma and HCC cell lines and consistently detected a significant association between the protein levels of gankyrin and IGFBP-5 in HCC specimens. In the proximal promoter region of the IGFBP-5 gene, there are several putative transcription-factor–binding sites including those for AP-2, c-Myb, C/EBP, and NF-1, and responsive elements to prostaglandin E_2, cyclic adenosine monophosphate, progesterone/retinoic acid, and Akt.[28] Whether the effect of gankyrin on IGFBP-5 expression is mediated by these factors is unknown.

The IGFBPs bind IGFs with high affinity, and they are able to enhance or inhibit the activity of IGFs in a cell-specific and tissue-type–specific manner.[28] In addition, IGFBPs have IGF-independent effects. There are several reports on the relationship between the IGF axis and HCC.[29-31] IGFBP-3 is the most abundant IGFBP present in noncancerous liver tissue and could serve as a negative regulator of cell proliferation in human HCCs.[32] Although the presence of IGFBP-5 in numerous tumors and cell lines has been demonstrated, its expression and significance in human HCC have not been documented. We found positive staining for IGFBP-5 in 42% of HCCs, and the positivity correlated with absence of portal venous invasion, low TNM stage, and small tumor size. Although not statistically significant, patients with IGFBP-5–positive HCCs tended to survive longer than those with IGFBP-5–negative HCCs. These findings are essentially similar to those observed for gankyrin. Regarding the effect of IGFBP-5 on cell proliferation, there are contradictory findings.[28] In breast cancer cells, many studies have reported inhibition of growth, but there are some indicating a stimulatory effect.[33] IGFBP-5 is up-regulated in involuting prostate but is also implicated in growth stimulation of prostate tumor cells.[34] We found that down-regulation of IGFBP-5 suppresses growth of Huh-7 HCC cells. Thus, these findings are consistent with a notion that high expression of IGFBP-5 and gankyrin play oncogenic roles in HCCs of early clinical stages. Clarification of the exact roles played by them will shed more light on the molecular mechanisms of human hepatocarcinogenesis and lead to development of new therapeutic and preventive strategies.

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Original Article

Activation of B-Myb by E2F1 in hepatocellular carcinoma

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Aim: Deregulation of E2F1 transcriptional activity is observed in a variety of cancers, including hepatocellular carcinoma (HCC). The aim of the present study is to identify transcriptional target genes of E2F1 in HCC.

Methods: We determined expression levels for *E2F1* and ten candidate genes thought to be targets of E2F1 in primary HCCs using a real-time quantitative reverse transcription-PCR assay. Following small interfering RNA (siRNA)-mediated knockdown of *E2F1* in HCC cell lines, we quantified mRNA levels of the candidate E2F1 target genes.

Results: *E2F1* was significantly over-expressed in 41 primary HCCs as compared to non-tumorous liver tissues. Among the candidates, *MYBL2*, whose product is the transcriptional factor B-Myb, which is involved in controlling cell-cycle progression and apoptosis, was significantly over-expressed in primary HCCs. Additionally, expression levels of *MYBL2* correlated with those of *E2F1*. Knockdown of *E2F1* resulted in a decrease in expression of *MYBL2*. A copy-number gain for *MYBL2* was observed in 36 of 66 primary HCCs, suggesting that *MYBL2* expression is up-regulated by amplification in addition to being regulated by E2F1. Moreover, siRNA-mediated knockdown of *MYBL2* led to reduced expression of *CDC2* (which encodes CDC2), cyclin A2 (*CCNA2*), and topoisomerase II α (*TOP2A*), implicating these genes in the cell cycle and suggesting that they may be downstream targets of B-Myb.

Conclusion: *MYBL2* is a probable transcriptional target of E2F1 in HCC and may therefore be a useful biomarker for diagnosis and an attractive target for molecular therapies useful to treat HCC.

Key words: CCNA2, CDC2, E2F1, hepatocellular carcinoma, MYBL2, TOP2A

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common malignancy in the world and is estimated to result in approximately half a million deaths annually.[1] Several risk factors for HCC have been reported, including infection with hepatitis B virus (HBV), hepatitis C virus (HCV), dietary aflatoxin, alcohol consumption, and diabetes.

Deregulation of E2F transcriptional activity as a result of alterations in the $p16^{INK4a}$-cyclin D1-Rb pathway is a hallmark of human cancer. E2Fs comprise a family of related factors that control the expression of genes important for cell-cycle progression as well as other processes such as apoptosis, DNA repair, and differentiation.[2,3] There are now eight known human E2F family genes: *E2F1-E2F8*. The E2F1 through to E2F6 proteins dimerize with one of three DP proteins (DP1, DP2/3, or DP4) to form functional transcriptional factor complexes that can bind DNA with high affinity. The function of the E2F-DP heterodimer is thought to be determined primarily by which E2F is present in the heterodimeric complex.

Our earlier studies identified *TFDP1*, which encodes DP1, as a probable target within a 13q34 amplicon that is frequently detected in HCCs[4] and esophageal squamous cell carcinomas.[5] Elevated expression of *TFDP1* was associated with large HCC tumor size and down-regulation of *TFDP1* inhibited growth of HCC cells.[6]

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