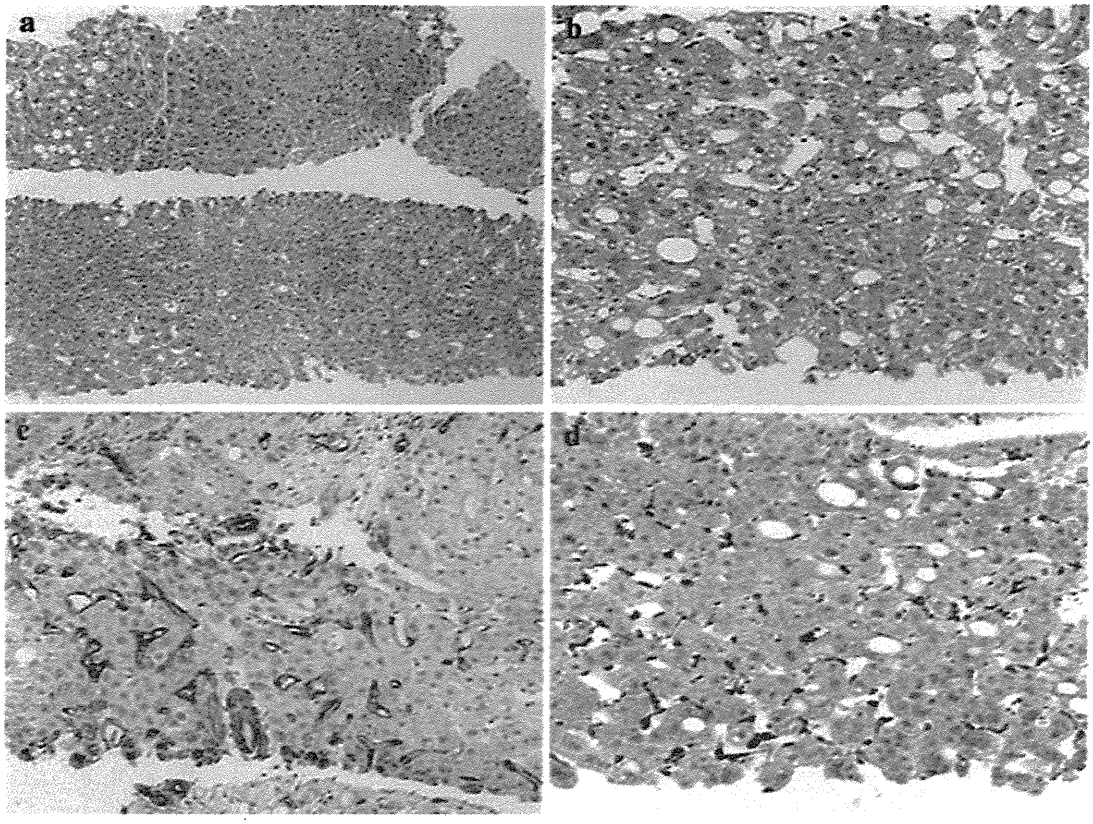

Fig. 3 Pathological findings of a liver biopsy in case 1. In comparison to the background liver tissue, the nodules showed a slightly increased cell density, scar-like fibrosis, and sinusoidal dilatation. Part of the nodule showed steatosis (**a**, **b**). The double immunostain for both CD34 and alpha-smooth muscle actin showed a diffuse staining pattern in the sinusoidal endothelial cells, suggesting sinusoidal capillarization and unpaired arteries (**c**). Several CD68-positive Kupffer cells were detected in this nodule (**d**)

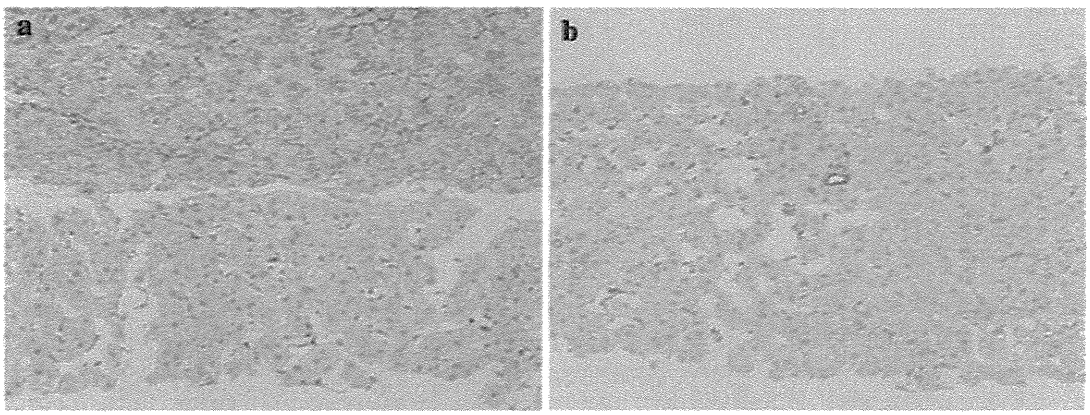

Fig. 4 The expression of OATP1B3 was observed predominantly in the cell membrane in the background liver in case 1 (**a**). However, part of this nodular lesion did not show any expression of OATB1B3 (**b**)

several CD68-positive Kupffer cells were observed in this nodule. These findings suggested a FNH-like nodule, arising from alcoholic cirrhosis, rather than a well-differentiated HCC.

Moreover, no immunostaining of OATB1B3 was observed in this nodule. These findings were consistent with the low intensity observed in the hepatobiliary phase of MRI (Fig. 7).

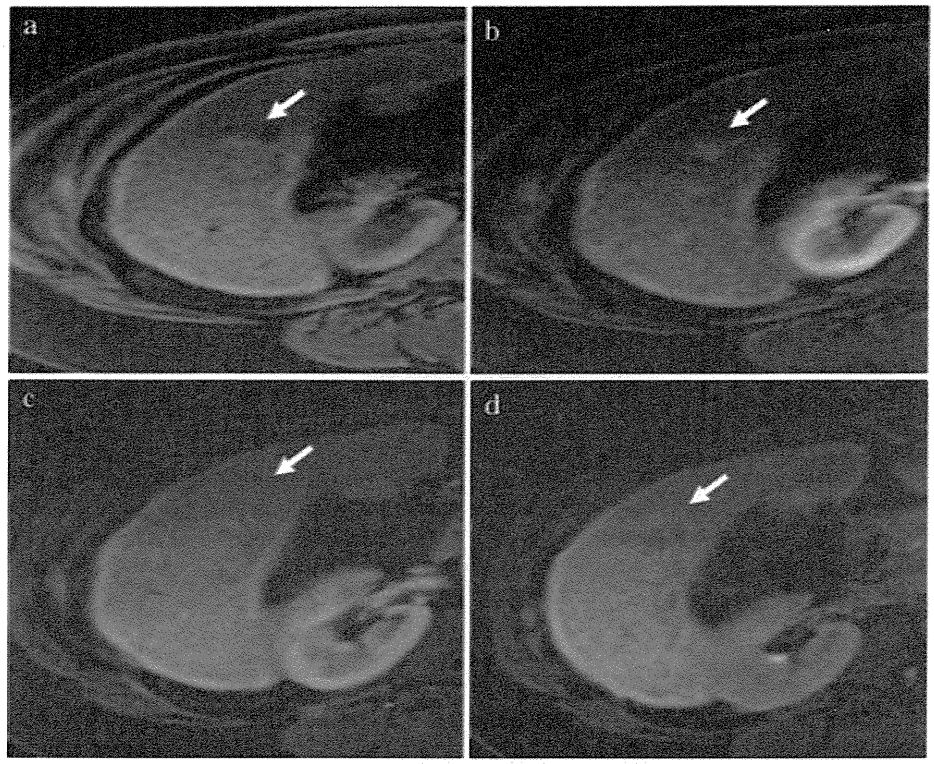

Fig. 5 Magnetic resonance imaging findings in case 2. The nodular lesion in liver S5 showed a high intensity at the precontrast T1 weighted image (**a**),was slightly enhanced at the early contrast phase of (**b**), was washed out at the portal phase (**c**), and had a defect in the hepatobiliary phase (**d**) (3D-GRE FS-T1W1, TR 4.30, TE 1.61, FA10)

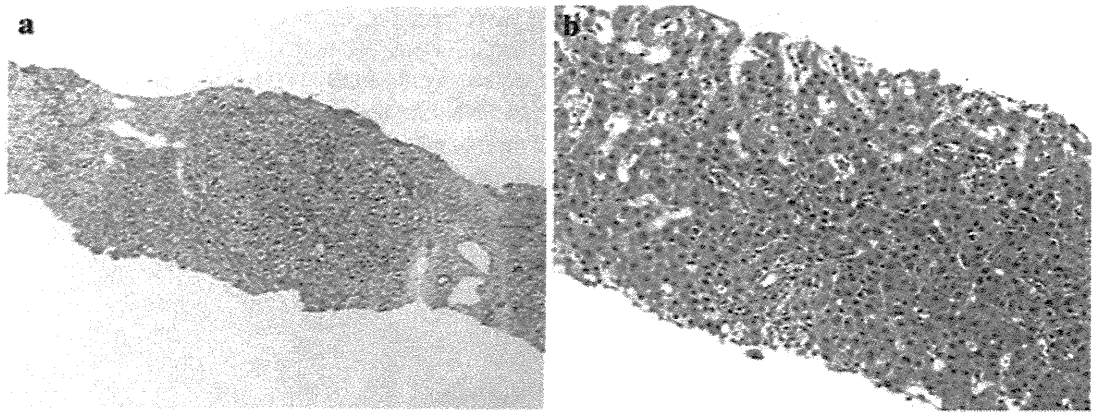

Fig. 6 Pathological findings of a liver biopsy in case 2. The background liver showed cirrhosis (**a**). The nodule showed a slightly increased cell density and sinusoidal dilatation in comparison to the background liver tissue (**b**)

Discussion

Although the International Working Party classified nodular hepatocellular lesions into several main classes, including regenerative lesions and dysplastic or neoplastic lesions [5], the most important clinical concern is the ability to distinguish between a regenerative lesion and a tumor lesion.

In the two current cases, HCC was first suspected from the imaging studies, which revealed a hypervascular tumor, although hyperplastic nodules arising from alcoholic liver cirrhosis were considered in the differential diagnoses.

Regarding the tumor markers, PIVKA II was elevated in case 1 and AFP was elevated in case 2. AFP is a well-known tumor marker of HCC, and its diagnostic efficacy has been confirmed; however, its serum levels are also observed to increase in liver diseases such as chronic hepatitis and cirrhosis. Conversely, the serum PIVKA II level is significantly elevated in alcoholic liver disease compared to viral liver disease, although the mechanism of

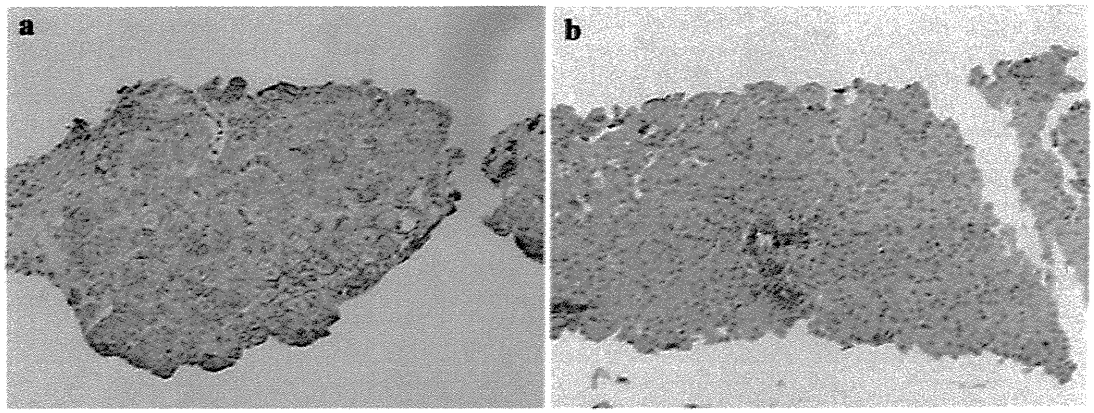

Fig. 7 The cell membrane of the background liver showed diffuse OATP1B3 in case 2 (**a**). This nodular lesion did not show any OATB1B3 expression (**b**)

the elevation of PIVKA II has not been clarified [6]. Ethanol intake and the following vitamin K deficiency may act, in part, due to an increased serum PIVKA II level in alcoholic liver disease. Taken together, tumor markers are not sufficient to differentiate between well-differentiated HCCs and FNH-like nodules arising in patients with alcoholic liver cirrhosis.

In case 1, the dynamic CT showed multiple nodular lesions with a delayed washout. In case 2, the dynamic CT showed multiple nodular lesions with arterial enhancement and a delayed washout. These findings were highly suggestive of a well-differentiated HCC. In most previous reports, these FNH-like nodules arising in patients with alcoholic liver cirrhosis lesions showed that the dynamic CT imaging resembles HCC, and furthermore, these two lesions are very difficult to differentiate from one another [1, 7–10].

Moreover, in case 1 the nodule in S2 showed encapsulation. While encapsulation is one of the typical findings in HCC, all FNH-like nodules in alcoholic liver cirrhosis are also reported to be encapsulated [1].

Recently, it has been reported that Gd-EOB-DTPA MRI has been helpful for the detection of small HCCs. In the present cases, most of the nodules showed defects in the hepatobiliary phase, suggesting HCC. Therefore, it is difficult to differentiate these hyperplastic nodules from well-differentiated HCCs using Gd-EOB-DTPA MRI alone. The difference in contrast following the administration of Gd-EOB-DTPA depends on the expression levels of the uptake transporter OATP1B3 [11] and excursion transporter (MRP2) [12]. The nodule in S2 showed partly positive expression of OATP1B3 in case 1. This finding was consistent with the MRI that showed slightly low intensity in the hepatobiliary phase. The S2 nodule in case 2 showed no OATP1B3 expression. These findings suggest that low intensity in the hepatobiliary phase depends on the lack of OATP1B3 expression in FNH-like hyperplastic nodules.

Moreover, some reports have shown the Gd-EOB-DTPA uptake to be associated with pericellular fibrosis in nonalcoholic steatohepatitis [13, 14]. Pericellular fibrosis is the one of characteristic features of alcoholic liver disease. Therefore, the poor uptake of Gd-EOB-DTPA in our cases may depend on the degree of pericellular fibrosis in the nodule.

We reported on two patients with FNH-like nodules arising from alcoholic cirrhotic livers, which revealed defects by Gd-EOB-DTPA MRI. The differential diagnosis of FNH-like nodules in patients with alcohol-induced cirrhosis and well-differentiated HCCs is very difficult with tumor marker and imaging studies alone, such as dynamic CT and Gd-EOB-DTPA MRI. Therefore, histological confirmation is required when nodules arising in patients with alcoholic cirrhosis are encountered.

References

1. Nakashima O, Kurogi M, Yamaguchi R, Miyaaki H, Fujimoto M, Yano H, et al. Unique hypervascular nodules in alcoholic liver cirrhosis: identical to focal nodular hyperplasia-like nodules? J Hepatol. 2004;41:992–8.
2. Vogl TJ, Kummel S, Hammerstingl R, Schellenbeck M, Schumacher G, Balzer T, et al. Liver tumors: comparison of MR imaging with Gd-EOB-DTPA and Gd-DTPA. Radiology. 1996; 200:59–67.
3. Vogl TJ, Hammerstingl R, Schwarz W, Kummel S, Muller PK, Balzer T, et al. Magnetic resonance imaging of focal liver lesions. Comparison of the superparamagnetic iron oxide resovist versus gadolinium-DTPA in the same patient. Invest Radiol. 1996; 31:696–708.
4. Frericks BB, Loddenkemper C, Huppertz A, Valdeig S, Stroux A, Seja M, et al. Qualitative and quantitative evaluation of hepatocellular

carcinoma and cirrhotic liver enhancement using Gd-EOB-DTPA. AJR Am J Roentgenol. 2009;193:1053–60.
5. Terminology of nodular hepatocellular lesions. International Working Party. Hepatology. 1995;22:983–93.
6. Ohhira M, Ohtake T, Saito H, Ikuta K, Tanaka K, Tanabe H, et al. Increase of serum des-gamma-carboxy prothrombin in alcoholic liver disease without hepatocellular carcinoma. Alcohol Clin Exp Res. 1999;23:67S–70S.
7. Kim SR, Maekawa Y, Imoto S, Sugano M, Kudo M. Hypervascular liver nodules in heavy drinkers of alcohol. Alcohol Clin Exp Res. 2004;28:174S–80S.
8. Kim SR, Maekawa Y, Ninomiya T, Imoto S, Matsuoka T, Ando K, et al. Multiple hypervascular liver nodules in a heavy drinker of alcohol. J Gastroenterol Hepatol. 2005;20:795–9.
9. Moon JH, Ahn CM, Chung HS, Ahn SH, Park YN. A case of hypervascular hyperplastic nodules in a patient with alcoholic liver cirrhosis. Yonsei Med J. 2006;47:881–6.
10. Kim SR, Imoto S, Ikawa H, Ando K, Mita K, Shimizu K, et al. Focal nodular hyperplasia-like lesion with venous washout in alcoholic liver cirrhosis. Intern Med. 2008;47:1899–903.
11. Narita M, Hatano E, Arizono S, Miyagawa-Hayashino A, Isoda H, Kitamura K, et al. Expression of OATP1B3 determines uptake of Gd-EOB-DTPA in hepatocellular carcinoma. J Gastroenterol. 2009;44:793–8.
12. Pascolo L, Petrovic S, Cupelli F, Bruschi CV, Anelli PL, Lorusso V, et al. Abc protein transport of MRI contrast agents in canalicular rat liver plasma vesicles and yeast vacuoles. Biochem Biophys Res Commun. 2001;282:60–6.
13. Sonoda A, Nitta N, Ohta S, Nitta-Seko A, Tsuchiya K, Nagatani Y et al. The possibility of differentiation between nonalcoholic steatohepatitis and fatty liver in rabbits on Gd-EOB-DTPA-enhanced open-type MRI scans. Acad Radiol.2011;18:525–9.
14. Tsuda N, Okada M, Murakami T. New proposal for the staging of nonalcoholic steatohepatitis: evaluation of liver fibrosis on Gd-EOB-DTPA-enhanced MRI. Eur J Radiol.2010;73:137–42.

Clinical characteristics of hepatocellular carcinoma in elderly patients

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Abstract. The incidence of hepatocellular carcinoma (HCC) in elderly patients in Japan has been on the increase. The aim of the present study was to evaluate the impact of aging on the clinicopathological findings and the survival of HCC patients. A total of 624 patients with HCC were examined in this study. The patients were classified according to their age at the time of diagnosis: one group comprised younger patients (<75 years; n=544) and the second comprised elderly patients [(≥75 years; n=80, (12%)]. Results showed that there were significantly more female patients (younger:elderly, 22:36; p=0.005), normal livers (younger:elderly, 0.3:6%; p=0.0002), non-viral HCC (younger:elderly, 11:31%; p<0.001) and solitary tumors (younger:elderly, 53:76%; p=0.0008) in the elderly group. Five out of seven (71%) non-B non-C (NBNC) HCC patients who developed HCC in the normal liver were elderly patients. Survival between the younger and elderly HCC groups was not significantly different (younger:elderly, 4.38:3.45 years; p=0.665). Additionally, elderly HCC patients had fewer tumors, more mild underlying liver damage, and more frequent NBNC HCC. Their prognosis was not necessarily poorer than that of the younger HCC patients. Additionally, it appears that elderly patients develop HCC even without fibrosis. Therefore, aging may be a factor affecting hepatocarcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers (1,2), with an estimated half a million cases annually, worldwide. Although HCC is generally diagnosed in middle-aged and elderly individuals, the age distribution of HCC varies according to etiology. The differences in age at the time of diagnosis of HCC affect the treatment strategy.

The Japanese population has one of the longest average life spans, and the size of the aged population has been increasing rapidly. As a result, the prevalence of elderly patients with HCC has increased (3-5). There is some controversy regarding whether aging plays a role in the factors and survival of patients with HCC. Previous studies reported that the long-term survival of younger HCC patients is similar to that of elderly patients (6,7). On the other hand, it has been reported that elderly HCC patients tended to have a poorer prognosis (8).

A recent increase in the number of elderly HCC patients in Japan has been reported (4,9,10). However, the impact of aging on the emergence of HCC has yet to be adequately investigated. Therefore, the aim of the present study was to investigate the effect of aging on the clinicopathological findings and the survival of HCC patients.

Patients and methods

Patients. A total of 624 patients presenting with HCC at the Department of Gastroenterology and Hepatology, Nagasaki University School of Medicine, Japan, were recruited for this study, between October 1981 and October 2007. The diagnosis of HCC was based on α-fetoprotein (AFP) levels, des-γ-carboxy prothrombin (DCP) levels, imaging studies including ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG) and/or liver biopsy. The diagnosis of chronic liver disease and liver cirrhosis was based on the level of platelets and imaging studies and/or liver histology. The patients were classified into two groups according to their age at the time of diagnosis: a younger group (<75 years; n=544) and an elderly group (≥75 years; n=80).

Etiology of HCC. A diagnosis of chronic hepatitis C virus (HCV) infection was based on the presence of HCV antibodies (microparticle enzyme immunoassay; Abbott Laboratories, Tokyo, Japan) and HCV-RNA detected by polymerase chain reaction (PCR), whereas the diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBs/Ag) (enzyme-linked immunosorbent assay; Abbot

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Key words: hepatocellular carcinoma, aging, non-viral hepatocellular carcinoma

Table I. Patient characteristics.

Characteristics	
Age (years)	63.9±9.8
Gender, male : female	478:146
BMI	22.6±3.2
Normal : CH : LC	7:120:497
Child-Pugh grade	6.3±1.6
NBNC : HBV : HCV	74:139:430:19
Tumor diameter (cm)	4.3±3.5
No. of tumors	2.8±3.1

BMI, body mass index; CH, chronic hepatitis; LC, liver cirrhosis, NBNC, non-B non-C; HBV, hepatitis B virus; HCV, hepatitis C virus.

Table II. Comparison of the patient backgrounds.

	<75 Years old	≥75 Years old	p-value
	544 Cases	80 Cases	
Gender (female)	117 (22%)	29 (36%)	0.0050
Normal liver	2 (0.3%)	5 (6%)	0.0002
Liver cirrhosis	440 (80%)	57 (71%)	0.0450
Child-Pugh grade	6.3±1.7	6.0±2.2	0.1650
Prothrombin time (%)	77±19	79±24	0.4600
Bilirubin (mg/dl)	1.5±2.4	1.0±0.7	0.1080
Albumin (g/dl)	3.8±3.2	3.6±0.5	0.7380

Table III. Comparison of risk factors for hepatocellular carcinoma.

	<75 Years old	≥75 Years old	p-value
	544 Cases	80 Cases	
HBsAg-positive	131 (24%)	8 (10%)	0.004
HCVAb-positive	381 (70%)	49 (61%)	0.112
NBNC	59 (11%)	25 (31%)	0.001
Diabetes mellitus	152 (28%)	22 (28%)	0.934
Alcohol consumption	117 (22%)	10 (12%)	0.085

HBsAg, hepatitis B surface antigen; HCVAb, Hepatitis C antibody; NBNC, non-B non-C.

Laboratories). The history of alcohol intake was noted from medical records. Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g of pure ethanol over a period of >10 years.

Statistical analysis. The SPSS 9.0 for Windows statistical software program was used to assess correlations among multiple variables. When appropriate, clinical and laboratory data were compared with a χ^2 analysis, the Student's t-test or the Mann-Whitney test. The survival from time of diagnosis of HCC was analyzed using the Kaplan-Meier method and compared using the log-rank method. P<0.05 was considered to be statistically significant.

Table IV. Comparison of tumor characteristics and therapy for hepatocellular carcinoma.

	<75 Years old	≥75 Years old	p-value
	544 Cases	80 Cases	
Diameter (cm)	4.2±3.4	4.3±3.9	0.8250
No. of tumors	4.4±5.2	1.9±2.3	0.0060
Solitary cases	293 (53%)	56 (76%)	0.0008
TNM, stage I or II	338 (62%)	59 (73%)	0.0430
Surgical resection	68 (12.5%)	7 (9%)	0.3350
Local ablative therapy	144 (26%)	27 (33%)	0.1780
TACE	260 (47%)	40 (50%)	0.7130

TACE, transarterial chemoembolization.

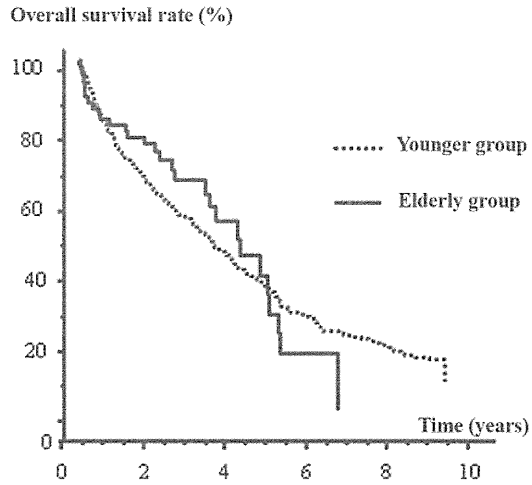

Figure 1. Kaplan-Meier model of the overall survival rate for the younger and elderly groups (younger HCC, 544 cases; elderly HCC, 80 cases). The overall survival between the younger and elderly HCC groups was not significantly different (p=0.665).

Results

Of the 624 patients, 80 (12%) patients were aged 75 years or older. The mean age of these older patients was 78.7±3.6. The clinical characteristics of the patients are shown in Table I. Significantly more patients in the elderly group were female (22:36%; p=0.005). The incidence of patients with liver cirrhosis was significantly higher, and the presence of a normal liver was significantly lower in the younger group than in the elderly group (80:71%; p=0.045; 0.3:6%, p=0.0002). No significant differences were observed in the protrobin time, total bilirubin, albumin or liver function as expressed by the Child-Pugh grade between the two groups (Table II).

Regarding viral status, the number of patients positive for HBsAg was significantly lower in the elderly group (24:10%, p=0.004), and the number of patients who were HBsAg and HCV antibody-negative [non-B non-C (NBNC)] was higher in the elderly group than in the younger group (11:31%, p=0.001).

In the NBNC HCC patients in the elderly group, 6 of 23 patients showed chronic hepatitis and 5 of 25 showed normal livers. No significant differences were found between the younger and elderly HCC groups with regards to alcoholism and diabetes mellitus (Table III).

No significant differences were noted in the tumor diameter between the younger and elderly groups. The number of HCC nodules was significantly lower in the elderly group than that in the younger group (4.4±5.2:1.9±2.3, p=0.006). The incidence of solitary cases and TNM stage I or II disease was significantly higher in the elderly group compared to that of the younger group (53:76%, p=0.0008; 62:73%, p=0.043). No significant differences were found between the younger and elderly HCC groups with regards to surgery, ablation therapy and transarterial chemoembolization (TACE) (Table IV).

The overall survival rate between the younger and elderly HCC groups was not significantly different (p=0.665). The overall median survival for the younger group was 4.38 years, compared with 3.45 years for the elderly group (Fig. 1).

Discussion

Age at diagnosis has been shown to have significant prognostic value in certain types of cancer. Although the number of elderly patients with HCC is on the increase in Japan (3,4), the characteristics and prognosis of HCC in elderly patients has yet to be elucidated. In this study, patients with HCC aged 75 years or older were examined, and their clinicopathological characteristics were identified and compared to those of the younger patients.

There were more male patients presenting with HCC in the younger group as compared to the elderly patients; one of the reasons for this being the difference in viral status. In this study, HBV infection, which is more common in males (11,12), was lower in the elderly group than in the younger group. Moreover, males were more likely to be heavy drinkers.

The prevalence of a normal liver was higher, whereas that of liver cirrhosis was lower in the elderly group. Of note is that 5 of 23 (21%) patients with NBNC HCC in the elderly group had normal livers. Additionally, 5 of 7 patients whose HCC developed in a normal liver were in the elderly group.

Chronic inflammation and viral infection are considered to be significant risk factors for HCC, but the elderly patients recruited in this study had neither factor. A previous study reported that the telomere length in the liver is shortened, not only with the progression of fibrosis staging, but also with aging (13). Moreover, the reduction of telomere length has been reported to increase the risk of HCC (14). Thus, elderly patients may have shorter telomeres, predisposing them to develop HCC, even if chronic liver disease was not prevalent. Findings of various studies have suggested that aberrant DNA methylation is a crucial epigenetic alteration in HCC (15-17). Some of the aberrant methylation observed in human cancer may be a consequence of chronic viral inflammation (18,19). On the other hand, aberrant methylation is also observed in the normal aging process (20), and may contribute to the occurrence of HCC in elderly patients with normal livers.

In this study, the HBV infection rate was lower, while the NBNC rate was higher in the elderly group than that in the younger group. Previous reports have shown that the average age of diagnosis of HBV-related HCC is approximately 55 years of age, whereas that of HCV-related HCC is approximately 65 years of age, and that of NBNC HCC is approximately 70 years of age (3,4). In Japan, the predominant time of transmission of the hepatitis B virus is during the prenatal period. The subsequent genomic long interreactions from an early age may lead to hepatocarcinogenesis at a younger age in the infected individuals.

On the other hand, patients with non-alcoholic steatohepatitis (NASH)-related HCC are older at diagnosis than those with HCC related to HBV and HCV (21,22). These results suggest that some of the NBNC HCC are NASH-related HCC.

The number of HCC nodules was lower, and the prevalence of single nodule HCC was higher in the elderly group than that in the younger group. Two main types of HCC occurrence exist, the first of which occurs at the time of the initial diagnosis with multicenter occurrence, which is associated with the degree of underlying liver damage. In this study, the prevalence of liver cirrhosis in the elderly group was lower than that in the younger group. The mild underlying liver damage in the elderly group may be associated with the smaller number of tumors observed in these patients.

Since elderly patients had fewer tumors and milder underlying liver damage at the time of the initial diagnosis, a more favorable prognosis in the elderly group may be expected. In this study, the overall survival rate was not significantly different between the two HCC groups. Overall, the majority of the elderly patients experienced various comorbidities, including cardiovascular disease, respiratory disease and diabetes mellitus. Taken together, the causes of death unrelated to HCC may have affected the survival rate in the elderly group.

In conclusion, elderly HCC patients had fewer tumors, milder underlying liver damage, and more frequent NBNC HCC. Additionally, it appears that elderly patients develop HCC even without fibrosis. Aging may therefore be a factor affecting hepatocarcinogenesis.

References

1. Bosch FX, Ribes J, Diaz M and Cleries R: Primary liver cancer: worldwide incidence and trends. Gastroenterology 127: S5-S16, 2004.
2. El-Serag HB and Mason AC: Risk factors for the rising rates of primary liver cancer in the United States. Arch Intern Med 160: 3227-3230, 2000.
3. Kiyosawa K, Umemura T and Ichijo T, *et al*: Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 127: S17-S26, 2004.
4. Taura N, Hamasaki K, Nakao K , *et al*: Aging of patients with hepatitis C virus-associated hepatocellular carcinoma: long-term trends in Japan. Oncol Rep 16: 837-843, 2006.
5. Kiyosawa K and Tanaka E: Characteristics of hepatocellular carcinoma in Japan. Oncology 62 (Suppl 1): 5-7, 2002.
6. Ng IO, Ng MM, Lai EC and Fan ST: Pathologic features and patient survival in hepatocellular carcinoma in relation to age. J Surg Oncol 61: 134-137, 1996.
7. Lam CM, Chan AO, Ho P, *et al*: Different presentation of hepatitis B-related hepatocellular carcinoma in a cohort of 1863 young and old patients - implications for screening. Aliment Pharmacol Ther 19: 771-777, 2004.

8. Falkson G, Cnaan A, Schutt AJ, Ryan LM and Falkson HC: Prognostic factors for survival in hepatocellular carcinoma. Cancer Res 48: 7314-7318, 1988.
9. Ohishi W, Kitamoto M, Aikata H, *et al*: Impact of aging on the development of hepatocellular carcinoma in patients with hepatitis C virus infection in Japan. Scand J Gastroenterol 38: 894-900, 2003.
10. Hamada H, Yatsuhashi H, Yano K, *et al*: Impact of aging on the development of hepatocellular carcinoma in patients with post-transfusion chronic hepatitis C. Cancer 95: 331-339, 2002.
11. Chen CJ, Yang HI, Su J, *et al*: Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. Jama 295: 65-73, 2006.
12. Chen CL, Yang HI, Yang WS, *et al*: Metabolic factors and risk of hepatocellular carcinoma by chronic hepatitis B/C infection: a follow-up study in Taiwan. Gastroenterology 135: 111-121, 2008.
13. Aikata H, Takaishi H, Kawakami Y, *et al*: Telomere reduction in human liver tissues with age and chronic inflammation. Exp Cell Res 256: 578-582, 2000.
14. Isokawa O, Suda T, Aoyagi Y, *et al*: Reduction of telomeric repeats as a possible predictor for development of hepatocellular carcinoma: convenient evaluation by slot-blot analysis. Hepatology 30: 408-412, 1999.
15. Yu J, Ni M, Xu J, *et al*: Methylation profiling of twenty promoter-CpG islands of genes which may contribute to hepatocellular carcinogenesis. BMC Cancer 2: 29, 2002.
16. Lee S, Lee HJ, Kim JH, Lee HS, Jang JJ and Kang GH: Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. Am J Pathol 163: 1371-1378, 2003.
17. Yang B, Guo M, Herman JG and Clark DP: Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. Am J Pathol 163: 1101-1107, 2003.
18. Ushijima T: Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 5: 223-231, 2005.
19. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE and Baylin SB: Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 7: 536-540, 1994.
20. Huja N, Li Q, Mohan AL, Baylin SB and Issa JP: Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res 58: 5489-5494, 1998.
21. Hashimoto E, Yatsuji S, Tobari M, *et al*: Hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. J Gastroenterol 44 (Suppl 19): 89-95, 2009.
22. Ascha MS, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF and Zein NN: The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. Hepatology 51: 1972-1978, 2010.

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Original Article

Suppressor of cytokine signal 3 and IL28 genetic variation predict the viral response to peginterferon and ribavirin

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Aim: The aim of this study was to investigate the relationship among the expression of suppressor of cytokine signaling 3 (SOCS 3) in the liver, the SNPs in the IL28B locus, and the outcome of interferon therapy.

Methods: Prior to interferon treatment, we immunostained 67 liver specimens from chronic hepatitis C (CHC) patients who were receiving peginterferon alpha-2b/ribavirin therapy for suppressor of cytokine signaling 3 (SOCS3), and compared the expression of SOCS3, IL28 polymorphisms and other clinical factors between the patients and compared their eventual outcomes.

Results: Significant differences between the low SOCS3 group and high SOCS3 group were found in age, as well as in the platelet, transaminase, gamma-glutamyl transpeptidase levels. The incidence of high SOCS3 was not significantly different between subjects with the TT genotype and the TG genotype (TT : TG = 71%:29%, $P = 0.250$). In a multivariate analysis, age (≥65 years old) (odds ratio 0.221 [0.120–0.966], $P = 0.045$), IL28B gene (genotype TT) (odds ratio 5.422 [1.254–23.617], $P = 0.024$) and SOCS3 (high) (odds ratio 0.308 [0.104–0.948], $P = 0.040$) were significant predictors of the interferon response. In patients with the TT genotype, those with low SOCS3 immunostaining showed a high sustained virological response (69%), while the sustained virological rate was low (27%) in the patients with high SOCS3 immunostaining.

Conclusions: Using a combination of the SOCS3 immunostained area in the liver and the expression of IL28B single nucleotide polymorphisms might be a useful predictor of hepatitis C virus clearance by interferon therapy.

Key words: hepatitis C virus, IL28B, interferon, suppressor of cytokine signaling 3

INTRODUCTION

APPROXIMATELY 200 MISSION people worldwide are infected with hepatitis C virus (HCV). In Japan, about 2 million people are chronically infected, and HCV is the leading cause of hepatocellular carcinoma (HCC). The current standard care for chronic hepatitis C (CHC) is a combination of peginterferon-α (PEG-IFN) and ribavirin. This treatment is effective in approximately 40–50% of CHC patients with a high viral load of genotype 1.[1–5] This therapy is costly and frequently associated with side effects. Therefore, predicting the outcome of interferon therapy is important.

Several factors, such as gender, body mass index, the presence of steatosis and liver fibrosis, drug adherence and viral factors including the serum quantity of HCV RNA and HCV genotype have been reported to be significantly associated with the treatment outcome.[2,6–11] Among viral factors, Akuta *et al.* recently reported that the substitution of the HCV core amino acid was a predictor for the effect of interferon and ribavirin combination therapy.[2,12] Among the host factors, recent reports showed that genetic variations near the IL28 gene (rs8099917, rs1297860) on chromosome 19 were predictors of the virological response to 48-week PEG-IFN plus ribavirin combination therapy in individuals

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with HCV, and also affected the clinical outcome, including spontaneous clearance of HCV.[13-15]

We previously reported that the expression of suppressor of cytokine signaling 3 (SOCS3), which is related to insulin resistance, impairs the response to interferon treatment and might be a useful predictor of HCV clearance by interferon therapy.[16]

In this study, we examined the relationship among the expression of SOCS 3 in the liver, single nucleotide polymorphisms (SNPs) in the IL28B locus, and the outcome of interferon therapy.

METHODS

NEEDLE BIOPSIES OF the liver were obtained from 67 patients with positive HCV antibodies prior to interferon treatment at Nagasaki University Hospital and National Hospital Organization (NHO) Nagasaki Medical Center. Twenty of 67 cases were also examined in a previous study.[16] All patients with genotype 1b received weekly injections of PEG-IFN. The clinical data of the patients are summarized in Table 1. Liver biopsy was performed by needle puncture for diagnostic purposes. The diagnosis of each case was independently confirmed histologically by liver pathologists according to the Japanese chronic hepatitis classification criteria (New Inuyama classification). According to these criteria, mild activity was defined as A0 or A1, severe activity as A2 or A3, mild fibrosis as F0 or F1, and severe fibrosis as F2, F3, or F4. Fatty changes in >5% of all areas were defined as steatosis.

Table 1 Clinical backgrounds of the patients

Age	56.8 ± 9.3
Gender	Male : Female = 37:30
BMI (kg/m^2)	23.5 ± 2.9
Viral load (KIU/mL)	2320 ± 1519
White blood cell (/uL)	5074 ± 1713
Hemoglobin (mg/dL)	14.1 ± 1.3
Platelet (×10^3/uL)	167.3 ± 75.6
AST (IU/L)	77.1 ± 45.2
ALT (IU/L)	101.2 ± 56.3
γGTP (IU/L)	70.6 ± 65.5
HCV core 70 wild	40 cases
HCV core 91 wild	50 cases
Steatosis (>5%)	37 cases
A (0–1:2–3)	36:31
F (0~1:2–4)	22:45

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus.

All patients received PEG-IFN (Schering-Plough, Tokyo, Japan) + ribavirin (Schering-Plough, Tokyo, Japan) therapy for 48 weeks. The patients who were treated with a dose of PEG-IFN or ribavirin reduced by more than 20% were excluded from the study. PEG-IFN (1.5 μg/kg) was administered once per week, and the ribavirin dose was titrated according to body weight. A sustained virological response (SVR) was defined as undetectable HCV RNA at 6 months after the end of interferon treatment.

Of 38 patients who could not achieve an end-of-treatment response, 28 patients required a re-elevation of their viral loads regardless of the fact that the HCV-RNA levels were temporarily negative, and 10 patients did not achieve an HCV negative result during the entire treatment period.

SOCS3 immunohistochemistry

All tissue samples were fixed in 10% neutral buffered formalin and then embedded in paraffin, and 4 μm thick serial sections were cut from each paraffin block. In the immunohistochemical study, an anti-SOCS3 antibody (dilution 1:100, Affinity BioRegents, Golden, CO, USA) was used for SOCS3. Immunohistochemistry was performed with the labeled streptavidin biotinylate antibody (LSAB) method and a commercially available kit (Histofine, SAB-PO(R); Nichirei Corporation, Tokyo, Japan). The area immunostained for SOCS 3 was divided according to the number of immunoreactive cells per unit area. Immunoreactive cases were classified as those with less than 30% of the hepatocellular cells stained (low SOCS3 group) and those with 30% or more of the cells stained (high SOCS3 group), because our previous study showed that staining of more than 30% of the area was a significant predictor of viral clearance.[16]

Genetic variation near the IL28B gene

Genotyping for replication was performed by use of the Invader assay or direct sequencing. In this study, genetic variation near the IL28B gene (rs8099917), which was previously reported to be a predictor of the virological response was investigated.[13]

Statistical analysis

The SPSS 9.0 for Windows statistical software program was used to assess correlations among multiple variables. When appropriate, clinical and laboratory data

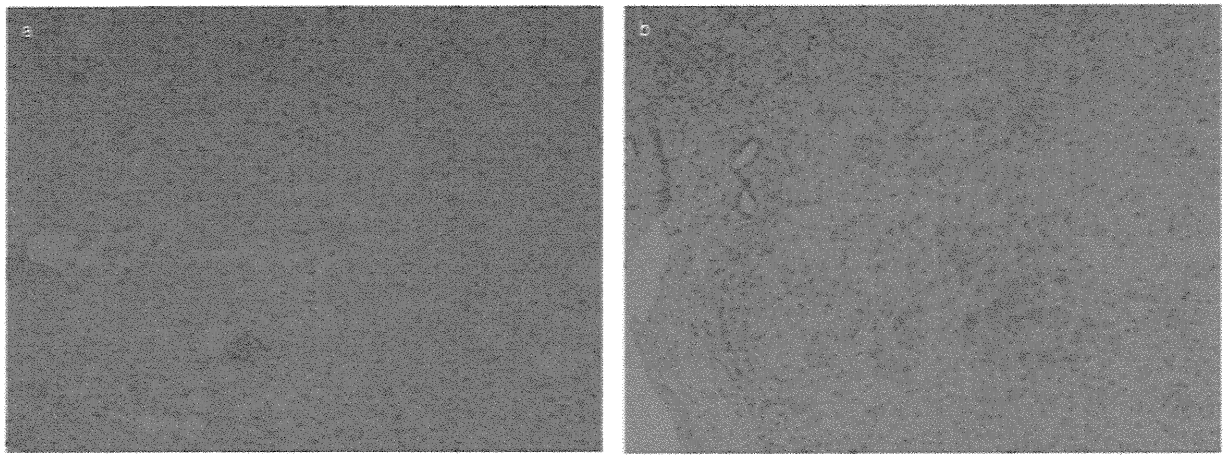

Figure 1 (a) This case showed less than 5% suppressor of cytokine signaling 3 (SOCS3) immunostained areas (low immunostaining). (b) This cases showed about 50% SOCS3 immunostaining areas (high immunostaining).

were compared with the Student's *t*-test or the Mann–Whitney test. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Immunostaining of SOCS3 in the liver (Figs 1,2)

IMMUNOSTAINING FOR SOCS3 was mainly seen in the periportal area. Less than 30% SOCS3 immunostained areas were found in 36 cases (54%) and areas with 30% or more immunostaining for SOCS3 were found in 31 cases (46%).

The frequency and distribution of the SOCS3 expression are shown in (Fig. 2)

Correlation between SOCS3 immunostaining and clinicopathlogical factors

A significant difference between low and high SOCS3 groups was found in age (low : high = 54.5 ± 9.8:59.5 ± 8.1, $P = 0.028$), the levels of platelets (low : high = 189.5 ± 90.0:141.6 ± 41.3, $P = 0.009$), aspartate aminotransferase (AST) (low : high = 94.5. ± 56.0:62.1 ± 33.5, $P = 0.003$), alanine aminotransferase; (ALT) (low : high = 85.8 ± 52.4:119.0. ± 56.3, $P = 0.015$), gamma-glutamyl transpeptidase (γGTP) (low : high = 48.8 ± 53.5:94.7 ± 70.6, $P = 0.004$). The incidence of steatosis (low : high = 33%: 81%, $P = 0.001$), severe activity (low : high = 27%: 67%, $P = 0.001$) and sever fibrosis (low : high = 52%: 84%, $P = 0.006$) was significantly higher in the SOCS3 high

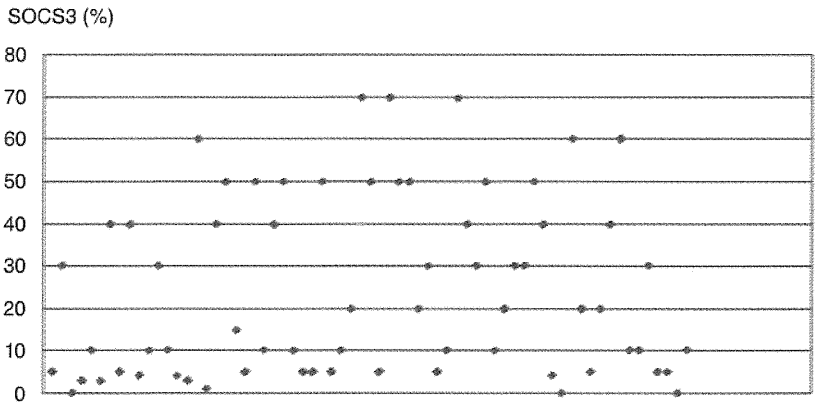

Figure 2 The distribution of the SOCS3 immunostaining area is shown.

Table 2 Comparison of the suppressor of cytokine signaling 3 (SOCS3) immunostaining groups

	SOCS3 high 31 cases	SOCS3 low 36cases	*P*-value
Age	59.5 ± 8.1	54.5 ± 9.8	0.028
Gender (male)	16 (53%)	21 (58%)	0.581
BMI (kg/m^2)	23.3 ± 2.2	23.6 ± 3.5	0.719
Viral load (KIU/mL)	2139 ± 1367	2475 ± 1950	0.427
White blood cell (/uL)	4935 ± 1386	5039 ± 1384	0.765
Hemoglobin (mg/dL)	14.1 ± 1.1	14.0 ± 1.3	0.570
Platelet (×10^3/uL)	141.6 ± 41.3	189.5 ± 90.0	0.009
AST (IU/L)	94.5. ± 56.0	62.1 ± 33.5	0.003
ALT (IU/L)	119.0 ± 56.3	85.8 ± 52.4	0.015
γGTP (IU/L)	94.7 ± 70.6	48.8 ± 53.5	0.004
Core 70 wild	17 (55%)	23 (63%)	0.451
Core 91 wild	23 (74%)	27 (75%)	0.939
Steatosis	25 (81%)	12 (33%)	0.001
Activity (severe)†	21 (67%)	10 (27%)	0.001
Fibrosis (severe)‡	26 (84%)	19 (52%)	0.006
IL28 TT rs8099917	22 (71%)	29 (80%)	0.358

†Severe activity was defined as A2 or A3.
‡Severe fibrosis was defined as F2, F3, or F4.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus.

immunostaining group than in the SOCS3 low immunostaining group. No significant difference was observed between the SOCS3 low and high groups in any of the other clinical factors (age, body mass index [BMI], viral load, white blood cell count, hemoglobin, substitution of the core 70, 91) (Table 2).

Comparison of SOCS3 expression and the genetic variation of IL28B gene

No significant difference in the genetic variation of the IL28 TT genotype was observed between the SOCS3 low and high immunostaining groups (low : high = 80%: 71%, $P = 0.250$).

Assessment of SOCS3 expression and genetic variation in IL28 as predictors of a sustained virological response

The age of patients in the non responder (NR) group was significantly higher than that in sustained virological response (SVR) group (SVR : NR = 52.3 ± 11.5: 59.6 ± 6.1, $P = 0.003$).

The incidence of the IL28 TT genotype was significantly lower, and that of SOCS3 high immunostaining group was significantly higher in the NR group than in the SVR group (Table 3).

As determined by a logistic regression analysis, the significant predictor of an SVR was high age (≥65 years old) (odds ratio 0.221 [0.120–0.966], $P = 0.045$), the IL28 TT genotype (odds ratio 5.422 [1.254–23.617], $P = 0.024$) and SOCS3 (high) (odds ratio 0.308 [0.104–0.948], $P = 0.040$) (Table 4).We found that two of nine (22%) patients with the IL28 TG genotype and SOCS3 high immunostaining showed a SVR, while one of seven (14%) patients with the IL28 TG genotype and SOCS3 low immunostaining, six of 22 (27%) patients with the IL28 TT genotype and SOCS3 high immunostaining, and 20 of 29 (69%) patients with the IL28 TG genotype and SOCS3 low immunostaining showed a SVR (Fig. 3).

DISCUSSION

RECENT IMPROVEMENTS IN the efficiency of antiviral therapy have led to approximately 50% of patients with HCV genotype 1 achieving sustained viral clearance.[1–5] However, some patients are refractory to interferon therapy. A recent study reported that the presence of genetic variation near the IL28B gene (rs8099917, rs1297860) can be used as a pretreatment predictor of virlogical response to a 48-week PEG-IFN plus combination therapy in patients with HCV geno-

Table 3 Factors associated with the response to peginterferon-α (PEG-IFN) and ribavirin

	SVR 29 cases	NR 38 cases	*P*-value
Age	52.8 ± 11.0	59.8 ± 6.4	0.002
Gender (male)	17 (58%)	20 (52%)	0.625
BMI (kg/m^2)	23.9 ± 3.1	22.9 ± 3.1	0.190
Viral load (KIU/mL)	2188 ± 1764	2420 ± 1689	0.587
White blood cell (/uL)	4816 ± 1427	5225 ± 1287	0.242
Hemoglobin (mg/dL)	14.1 ± 1.1	14.0 ± 1.3	0.626
Platelet (×10^3/uL)	176.5 ± 52.8	160.3 ± 89.2	0.350
AST (IU/L)	75.5 ± 36.1	78.3 ± 51.5	0.795
ALT (IU/L)	108.9 ± 56.8	95.3 ± 56.0	0.333
γGTP (IU/L)	63.9 ± 61.9	75.7 ± 68.6	0.464
Core 70 wild	20 (69%)	20 (53%)	0.176
Core 91 wild	21 (72%)	29 (71%)	0.173
IL28 TT rs8099917	26 (90%)	25 (65%)	0.022
steatosis	14 (47%)	23 (61%)	0.452
Activity (severe)†	10 (34%)	21 (64%)	0.091
Fibrosis (severe)‡	18 (62%)	27 (71%)	0.437
SOCS3 (Positive)	8 (27%)	23 (61%)	0.015

†Severe activity was defined as A2 or A3.
‡Severe fibrosis was defined as F2, F3, or F4.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; NR, non responder; SOCS3, suppressor of cytokine signal 3; SVR, sustained virological response.

type 1.[13–15] We previously reported that SOCS3 was a factor associated with the response to PEG-IFN treatment.[16] We compared these factors and clarified their usefulness as predictors of PEG-IFN plus combination therapy.

In the laboratory data from our patients, a significant difference between the groups with weak and strong SOCS3 staining was found in the level of AST, ALT, and platelets. These laboratory data suggested that the SOCS3 immunostained area was significantly associated with the presence of inflammation and the fibrosis stage. Indeed, in a pathological study, the inflammation and fibrosis stage were significantly different between the low and high SOCS3 immunostaining groups. This finding was consistent with our previous study that showed that the SOCS3 immunostained area was influenced by inflammation and the fibrosis stage.[16]

Table 4 Results of a multilogistic regression analysis

	Odds ratio	*P*-value
Age (>65 years)	0.221 (0.120–0.966)	0.045
IL28 TT	5.422 (1.254–23.617)	0.024
SOCS3 (low)	0.308 (0.104–0.948)	0.040

SOCS3, suppressor of cytokine signal 3.

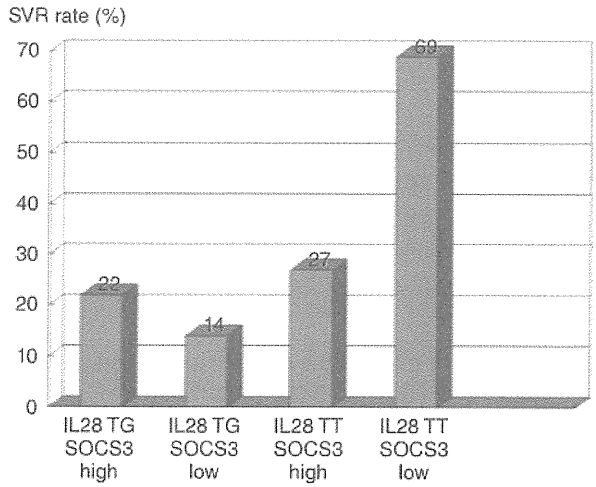

Figure 3 A total of 12.5% of patients with IL28 TG and suppressor of cytokine signaling 3 (SOCS3) high immunostaining showed a sustained virological response (SVR), 20% of patients with IL28 TG and SOCS3 low immunostaining, 31% of patients with IL28 TT and SOCS3 high immunostaining, and 68% of patients with IL28 TG and SOCS3 low immunostaining showed a SVR.

Moreover, a significant difference between the low and high SOCS3 groups was also found in the level of γGTP. Several previous reports showed that the level of γGTP was correlated with steatosis in the liver.[7,17] In this study, the presence of steatosis also was significantly different in the low and high SOCS3 immunostaining groups. Together with our results, these results demonstrated that the SOCS3 immunostained area in the liver was associated with obesity, insulin resistance, and hepatic steatosis.[18,19]

Although recent reports showed that genetic variation of IL28B was also associated with liver inflammation and fibrosis,[20] this was not associated with the SOCS3 immunostained area in the present study. The SOCS3 proteins are known for their role as negative regulators and inhibitors of Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling, where they mediate a classical negative feedback loop in the IFN-α/β receptor signaling pathway.[21,22] The mechanism that leads to the association between genetic variation of IL28B and the effect of interferon therapy is clear, because it has been demonstrated that IL28B inhibits hepatitis C virus replication through the JAK-STAT pathway.[23] Taken together, both the SOCS3 immunostained area and IL28B polymorphisms were associated with the JAK-STAT pathway, but the different factors might interfere with JAK-STAT signaling in different ways.

The NR rate to combination PEG-IFN plus ribavirin therapy in patients with the non-TT genotype was 10–20%. The value of NR for the prediction of the genetic variation of IL28B was therefore very high. On the other hand, the SVR rate in patients with the TG genotype was about 50%. The value of SVR prediction based only on the genetic variation of IL28B was therefore not as strong for this genotype.

The substitution of core amino acids was also reported to be a predictive factor for the response to interferon therapy and was significantly associated with the genetic variation of IL28B.[24] On the other hand, the SOCS3 immunostained area was independent of both of these factors. Thus, we suggested that using a combination of the SOCS3 immunostained area with the IL28B genotype can provide the best prediction of the response to PEG-IFN plus ribavirin therapy.

Indeed, in TT genotype patients, the SVR rate in the SOCS3 weak group was about 70%, and NVR rate in the SOCS3 low immunostained group was 27%. If a liver biopsy was performed, immunostaining for SOCS3 was easy, and provided a useful predictor of the response to interferon therapy.

Our study has some limitations. Our sample size was too small. Further large-scale studies are necessary to confirm the present results and to provide a better understanding of the interactions between the SOCS3 immunostained area and the genetic variation of IL28B.

In conclusion, a combination of the SOCS3 immunostained area in the liver and the assessment of the genetic variation of IL28B seem to be good predictors of the response to PEG-IFN plus ribavirin therapy.

REFERENCES

1 Mangia A, Ricci GL, Persico M *et al.* A randomized controlled trial of pegylated interferon alpha-2a (40 KD) or interferon alpha-2a plus ribavirin and amantadine vs interferon alpha-2a and ribavirin in treatment-naive patients with chronic hepatitis C. *J Viral Hepat* 2005; 12: 292–9.
2 Akuta N, Suzuki F, Kawamura Y *et al.* Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007; 46: 403–10.
3 Wedemeyer H, Wiegand J, Cornberg M, Manns MP. Polyethylene glycol-interferon: current status in hepatitis C virus therapy. *J Gastroenterol Hepatol* 2002; 17 (Suppl 3): S344–50.
4 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1493–9.
5 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; 352: 1426–32.
6 Bressler BL, Guindi M, Tomlinson G, Heathcote J. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. *Hepatology* 2003; 38: 639–44.
7 Yaginuma R, Ikejima K, Okumura K *et al.* Hepatic steatosis is a predictor of poor response to interferon alpha-2b and ribavirin combination therapy in Japanese patients with chronic hepatitis C. *Hepatol Res* 2006; 35: 19–25.
8 Zografos TA, Liaskos C, Rigopoulou EI *et al.* Adiponectin: a new independent predictor of liver steatosis and response to IFN-alpha treatment in chronic hepatitis C. *Am J Gastroenterol* 2008; 103: 605–14.
9 Yamada G, Iino S, Okuno T *et al.* Virological response in patients with hepatitis C virus genotype 1b and a high viral load: impact of peginterferon-alpha-2a plus ribavirin dose reductions and host-related factors. *Clin Drug Investig* 2008; 28: 9–16.

10 Iwasaki Y, Ikeda H, Araki Y *et al.* Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. *Hepatology* 2006; 43: 54–63.
11 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77–81.
12 Akuta N, Suzuki F, Hirakawa M *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 2a high viral load and virological response to interferon-ribavirin combination therapy. *Intervirology* 2009; 52: 301–9.
13 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41: 1105–9.
14 Thomas DL, Thio CL, Martin MP *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; 461: 798–801.
15 Suppiah V, Moldovan M, Ahlenstiel G *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; 41: 1100–4.
16 Miyaaki H, Ichikawa T, Nakao K *et al.* Predictive value of suppressor of cytokine signal 3 (SOCS3) in the outcome of interferon therapy in chronic hepatitis C. *Hepatol Res* 2009; 39: 850–5.
17 Ikai E, Ishizaki M, Suzuki Y, Ishida M, Noborizaka Y, Yamada Y. Association between hepatic steatosis, insulin resistance and hyperinsulinaemia as related to hypertension in alcohol consumers and obese people. *J Hum Hypertens* 1995; 9: 101–5.
18 Walsh MJ, Jonsson JR, Richardson MM *et al.* Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. *Gut* 2006; 55: 529–35.
19 Ueki K, Kondo T, Tseng YH, Kahn CR. Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. *Proc Natl Acad Sci U.S.A.* 2004; 101: 10422–7.
20 Abe H, Ochi H, Maekawa T *et al.* Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients. *J Hepatol* 2010; 53: 439–43.
21 Alexander WS. Suppressors of cytokine signalling (SOCS) in the immune system. *Nat Rev Immunol* 2002; 2: 410–6.
22 Yasukawa H, Sasaki A, Yoshimura A. Negative regulation of cytokine signaling pathways. *Annu Rev Immunol* 2000; 18: 143–64.
23 Zhang L, Jilg N, Shao RX *et al.* IL28B inhibits Hepatitis C virus replication through the JAK-STAT pathway. *J Hepatol* 2011; 55: 289–98.
24 Akuta N, Suzuki F, Hirakawa M *et al.* Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. *Hepatology* 2010; 52: 421–9.

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N-myc downstream regulated gene1/Cap43 overexpression suppresses tumor growth by hepatic cancer cells through cell cycle arrest at the G_0/G_1 phase

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ABSTRACT

N-myc downstream regulated gene-1 (NDRG1)/Cap43 regulates tumor growth and metastasis in various carcinomas. In this study we examined whether and how NDRG1/Cap43 modulates tumor growth by human hepatocellular carcinoma (HCC) cells. NDRG1/Cap43 cDNA was used to transfect HCC cell lines (KIM-1), and stable transfectants overexpressing NDRG1/Cap43 (KIM-1/Cap43) were obtained. Cell cycle analysis showed that KIM-1/Cap43 cells were arrested in the G_0/G_1 phase. Western blot analysis demonstrated an increase in p21 in KIM-1/Cap43 cells in culture under full confluency as compared with KIM-1/Mock. When KIM-1 cells, which are very low in NDRG1/Cap43 expression, were treated with mimosine, a G_0/G_1 cell cycle blocker, expression of NDRG1/Cap43 was induced in a dose dependent manner, together with p21 induction and CDK4 reduction. *In vivo*, KIM-1/Cap43 cells showed markedly decreased tumor growth rates compared with those of KIM-1/Mock. Immunohistochemical staining demonstrated markedly higher p21 labeling index in the KIM-1/Cap43 tumor than KIM-1/Mock tumor, and lower CDK4 and Ki-67 labeling index in the KIM-1/Cap43 than KIM-1/Mock. In order to confirm suppressive effects of NDRG1/Cap43, we further established a stable transfectant expressing NDRG1/Cap43 (HAK-1B/Cap43) using another HCC cell line, HAK-1B. Western blot analysis demonstrated an increase in p21 and a decrease in CDK4 in HAK-1B/Cap43 cells in culture under full confluency as compared with HAK-1B/Mock. HAK-1B/Cap43 also showed decreased tumor growth rates as compared with its control counterpart *in vivo*. NDRG1/Cap43 overexpression thus induced cell cycle arrest at the G_0/G_1 phase accompanied by increased p21 and decreased CDK4 expression in HCC cells. NDRG1/Cap43 might play a key role in the cell cycle control of G_0/G_1 in HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death in the world. HCC is frequently associated with hepatitis B virus (HBV) – or hepatitis C virus (HCV)-induced chronic hepatitis, α-1-antitrypsin

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deficiency, or the prolonged ingestion of alcohol or aflatoxin. The incidence of HCC differs in different geographical regions, being high in Asia and Africa. This is correlated with the frequency distribution of HBV or HCV infection. Although HCV infection rates in the United States have been historically lower than those in Asia and Africa, it is predicted that the spread of HCV infection will lead to an increase in the incidence of HCC [1]. Advances and improvements in the screening and treatment of patients at high risk for HCC have improved the prognosis of early-stage HCC [2]. However, the prognosis of advanced HCC remains extremely poor.

N-myc downstream-regulated gene-1 (NGRG-1)/calcium-associated protein 43 kDa (Cap43) is also known as Drg-1, RTP, and RIT42. NDRG1/Cap43 expression is regulated by nickel, cobalt, oxidative stress, hypoxia, phorbol esters, vitamin A, vitamin D, steroids, histone deacetylase-targeting drugs, lysophosphatidylcholine, oncogenes and tumor suppressor gene products [3–7]. NDRG1/Cap43 has the functions of keratinocyte differentiation [8], cell cycle regulation (4), myelin sheath maintenance [9], and the attenuation of hypoxic injury in human trophoblasts [10].

NDRG1/Cap43 is widely expressed in non-neoplastic tissue within the body [11]. The levels of NDRG1/Cap43 expression are lower in breast, colorectal, and prostate cancer cells than in non-neoplastic tissue, and those in prostate and pancreatic cancer are negatively correlated with the histological grade of malignancy [4,12–14]. NDRG1/Cap43 shows an inhibitory effect on the metastasis of prostate, colorectal, and breast cancer [12,15,16]. On the other hand, NDRG1/Cap43 serves as an indicator of a poor prognosis in cervical adenocarcinoma and HCC [17–19]. In our immunohistochemical study, NDRG1/Cap43 expression was increased in advanced HCC, and was overexpressed in patients with portal vein invasion or intrahepatic metastasis [19]. A study by Yan et al. has reported that the NDRG1/Cap43 knockdown by its cognate siRNA inhibited tumor cell proliferation and invasion by HCC cells [20]. In our present study, we examined whether NDRG1/Cap43 overexpression could affect tumor growth by HCC cells. The suppressive effect of NDRG1/Cap43 on the tumor growth by HCC cells is discussed in its close association with cell cycle arrest.

2. Materials and methods

2.1. Cell lines, cell culture and antibodies

Six human HCC cell lines originally established in our laboratory were used in this study: KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A and HAK-1B. These cell lines were previously confirmed to retain the morphological and functional characteristics of original HCC. The cells were grown in culture medium consisting of Dulbecco's modified Eagle medium (Nissui Seiyaku Co., Japan) supplemented with 5% heat-inactivated (56 °C, 30 min) fetal bovine serum (Bioserum, Victoria, Australia), 100 U/mL penicillin, 100 mg/mL streptomycin (Gibco BRL/Life Technologies, Inc., Gaithersburg, MD, USA), and 12 mM sodium bicarbonate in a humidified atmosphere of 5% CO_2 at 37 °C. The anti-NDRG1/Cap43 antibody was generated as described previously [14]. Other antibodies were purchased as follows: anti-cyclin A, cyclin B1, cyclin E, p16, p21, cyclin dependent kinase (CDK) 1/CDC2, CDK2, CDK4, epithelial growth factor receptor (EGFR), phospho-HER3(pHER3), IκBα, phospho-IκBα (pIκBα)(Cell Signaling Technology, Danvers, MA, USA); anti-CDC6 (Proteintech Group Inc., Chicago, IL, USA); anti-phospho-EGFR (pEGFR) (Calbiochem, Darmstadt, Germany); anti-HER2, phospho-HER2 (pHER2)(Upstate Biotechnology, Bedford, MA, USA); anti-βactin (Abcam, Cambridge, MA, USA); anti-Ki-67 (Novocastra, Newcastle, UK).

2.2. Expression vector construction and transfection

NDRG1/Cap43 cDNA was amplified by reverse transcription-PCR (RT-PCR) using the 5′ and 3′ primers 5′-CATGTCTCGGGAGATGCAGGATG-3′ and 5′-AGGCCGCCTAGCAGGAGACC-3′, respectively. Amplified NDRG1/Cap43 cDNA was ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and transferred to the pIRESneo2 expression plasmid (pIRESneo2-Cap43). Cells were transfected with pIRESneo2-Cap43 or pIRESneo2 using LipofectAMINE 2000 (Invitrogen) following the manufacturer's protocol. Stably transfected clones were established using G418 (Invitrogen) selection.

2.3. Effects of NDRG1/Cap43 on cell proliferation

The effect of NDRG1/Cap43 on the growth of cultured cells was examined with colorimetry using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay kit (Chemicon International Inc., Temecula, CA, USA). Briefly, KIM-1 cells showing NDRG1/Cap43 overexpression (KIM-1/Cap43) and the control (KIM-1/Mock) were seeded on 96-well plates (Nunc Inc., Roskild, Denmark). After culturing for Day 2, 3 and 4, the number of viable cells was measured with ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan) by setting the test wavelength at 570 nm and the reference wavelength at 630 nm. To keep the optimal density within the linear range, all experiments were performed while the cells were in the logarithmic growth phase.

2.4. Cell cycle analysis

KIM-1/Cap43 and KIM-1/Mock were cultured in serum-free culture medium for 24 h. After culturing in medium containing 10% FBS for 12 h, cells were labeled with 10 μmol/L BrdU for 30 min, fixed in 70% cold ethanol at 4 °C overnight, stained with anti-BrdU and propidium iodide (Sigma Chemical Co., St Louis, MO, USA), and then analyzed using FACScan (BD Bioscience, Bedford, MA, USA). Fixed cells were washed with PBS, subjected to double-stranded DNA denaturation treatment with 2 mol/L HCl at room temperature for 30 min, neutralized with 0.1 mol/L $Na_2B_4O_7$, washed twice with PBS, 0.5% Tween 20, and 0.5% BSA (PBS-T), incubated for 30 min at room temperature with 20 μL anti-BrdU antibody, washed with PBS-T, incubated for 30 min at room temperature with

4 μL fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin, and washed with PBS-T. DNA was counterstained with 5 μg/mL of propidium iodide for at least 20 min before flow cytometric analysis.

2.5. Western blot analysis

Cells were rinsed with ice-cold PBS and lysed in buffer containing 50 mmol/L Tris–HCl, 350 mmol/L NaCl, 0.1% Nonidet P-40, 5 mmol/L ethylenediaminetetraacetic acid, 50 mmol/L NaF, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mmol/L Na_3VO_4. Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide slab gel and blotted onto Immobilon membranes (Millipore Corporation, Bedford, MA, USA) as described previously [7]. After transfer, the membrane was incubated with blocking solution followed by primary antibody.

2.6. Morphological findings of culture cell

KIM-1/Cap43 or KIM-1/Mock cells were seeded on Lab-Tek Tissue Culture Chamber Slides (Nunc, Inc.), fixed in 4% paraformaldehyde for 20 min, and stained with H&E for light microscopic observation.

2.7. Mimosine, treatment in culture cell

KIM-1 cell, which is very low in NDRG-1/Cap43 expression, was treated with 10 μM to 400 μM mimosine (Sigma) for 24 h. Samples were subjected to Western blot analysis. KIM-1 was also treated without or with 200 μM and 400 μM mimosine for 24 h for cell cycle analysis.

2.8. Nude mouse xenograft experiment

Cells were suspended in sterile PBS at a concentration of 5×10^8 cells/mL, and 100 μL was injected subcutaneously into the right flank of the 4–10-week-old female BALB/c nu/nu athymic nude mice. The tumor size was measured in two directions using calipers, and the tumor volume (mm^3) was estimated using the equation: length × $(\text{width})^2 \times 0.5$. Measurement was performed every week, and changes in the average tumor volume were recorded. The mice were sacrificed after 7 weeks and the tumors were removed. Specimens were fixed in 10% formalin and embedded in paraffin blocks. Unstained 4-μm sections were cut from the blocks and stained with H&E for light microscopic observation. Immunohistochemistry for formalin-fixed paraffin-embedded sections was performed. Unstained sections were immunostained with antibodies against human NDRG1/Cap43, p21, CDK4 and Ki-67, following the method described previously [21]. All counts were performed by two independent observers (J.A. and M.Y.).

2.9. Effect of NDRG1/Cap43 in another HCC cell line overexpressing NDRG1/Cap43

To further confirm the effect of NDRG1/Cap43 overexpression on tumor growth by HCC cells *in vivo*, we established a stable NDRG1/Cap43 overexpressing cell line using different HCC cell line. HAK-1A and HAK-1B were previously established from a single HCC nodule of the well differentiated part and the dedifferentiated (poorly differentiated) part, respectively [22]. HAK-1B cells showing NDRG1/Cap43 overexpression (HAK-1B/Cap43) and the control (HAK-1B/Mock) were used following examinations. HAK-1B/Mock or HAK-1B/Cap43 cells (1×10^7) were used to inoculate 6–7-week-old male BALB/c nu/nu athymic nude mice subcutaneously, and the tumor diameters were measured every 3 days from Day 7. The tumor volume was estimated as previously described.

2.10. Statistical analysis

Data are expressed as the mean ± SD. Comparisons between groups were performed using Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. NDRG1/Cap43 overexpression inhibits the cell proliferation of HCC cell line KIM-1 in culture

We first compared the protein levels of NDRG1/Cap43 in four HCC cell lines. Out of the four cell lines, KYN-2 and KYN-3 showed comparable levels of NDRG1/Cap43 expression whereas there was no apparent expression of NDRG1/Cap43 in KIM-1 and KYN-1 (Fig. 1A). We next established cell line KIM-1/Cap43 by the transfection of NDRG1/Cap43 cDNA into KIM-1. KIM-1/Cap43 showed enhanced expression of NDRG1/Cap43 as compared to its parental mock transfectants, KIM-1/Mock and (Fig. 1B). We also compared the growth rates between mock and NDRG1/Cap43 transfectants of KIM-1 in the presence of 10% serum. KIM-1/Cap43 showed a significantly slower growth rate as compared with that of its parental counterpart (Fig. 1C). There were no apparent morphological differences between KIM-1/Cap43 and KIM-1/Mock (Fig. 1D).

3.2. NDRG1/Cap43 induced cell cycle arrest in the G_0/G_1 phase

We examined the cell cycle status by flowcytometric analysis.

There was relatively more cell population (60.7%) of KIM-1/Cap43 cells, in the G_0/G_1 phase than cell population (46.0%) of KIM-1/Mock cells (Fig. 2A). Since we have previously reported that NDRG1/Cap43 overexpression induced suppression of NF-κB pathway in human pancreas cancer cells [14,23], we examined the expression of IκBα and pIκBα in addition to EGFR family proteins. Expression protein levels of total and phosphorylated EGFR family proteins, IκBα and pIκBα were compared by Western blot analysis (Fig. 2B). There was no apparent difference in the expression level of EGFR, HER2 and HER3, and their phosphorylated proteins. By contrast the expression of IκBα and pIκBα was reduced in KIM-1/Cap43 as compared to KIM-1/Mock. Furthermore, Western blot analysis of cell cycle-related proteins revealed increased expression p21 in KIM-1/Cap43 under confluent culture condition. However, there was no difference in the protein levels of cyclin A, cyclin B1, cyclin E, p16, CDK1/CDC2, CDK2, CDK4 and CDC6 between KIM-1/Cap43 and KIM-1/Mock (Fig. 2C).

We next examined whether NDRG1/Cap43 overexpression was specifically associated with cell cycle at G_0/G_1 arrest. We examined the effect of a specific blocking reagent of cell cycle G_0/G_1, mimosine, on exponentially growing KIM-1 cells. Expression of NDRG1/Cap43 was markedly upregulated when exposed to various doses of mimosine at 200 μM and 400 μM for 24 h (Fig. 3A). Mimosine also induced expression p21 at

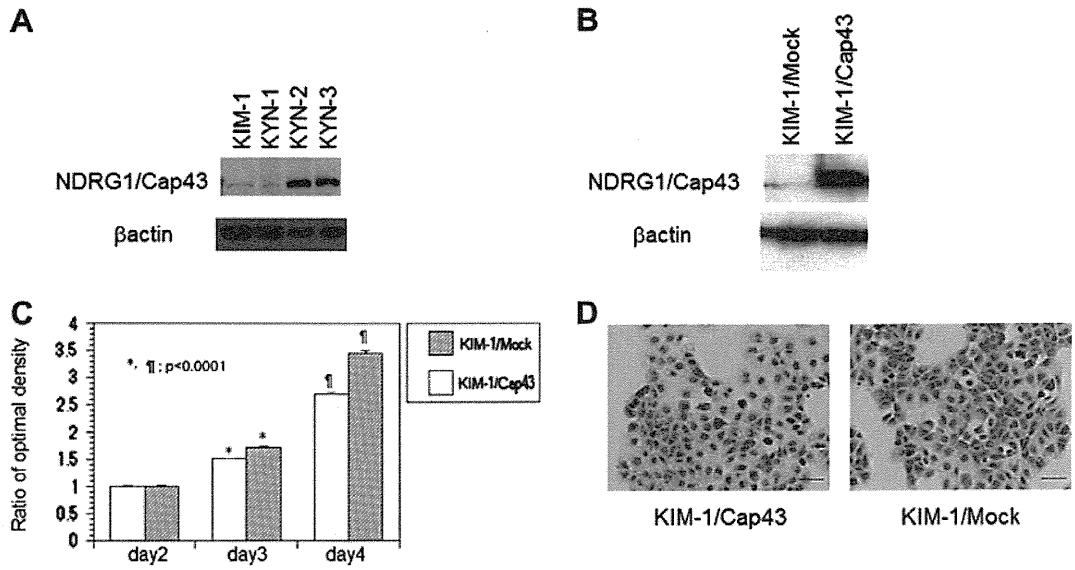

Fig. 1. Effect of NDRG1/Cap43 expression, cell proliferation, and morphological findings. (A) Expression of NDRG1/Cap43 in six HCC cell lines determined by Western blot analysis. Four cell lines out of six had NDRG1/Cap43 expression. (B) Expression of NDRG1/Cap43 in NDRG1/Cap43 and mock transfectants in KIM-1 cell line determined by Western blot analysis. (C) Comparison of cell growth between NDRG1/Cap43 and mock transfectants in KIM-1 cell line. Cell growth was measured at Day 2, 3, and 4 after seeding. Cell growth of NDRG1/Cap43 transfectant was significantly suppressed at 3 and 4 days compared with that of mock transfectant. The *Y*-axis shows the ratio of optimal density when that of Day 2 was 1.0. (D) There was no apparent morphological difference between NDRG1/Cap43 and mock transfectants. Scale bar indicates 50 μm.

200 μM and 400 μM and inhibited CDK4 expression at 400 μM (Fig. 3A). Cell cycle was specifically arrested at G_0/G_1 when KIM-1 cells were treated with 200 μM or 400 μM mimosine for 24 h (Fig. 3B).

3.3. A marked decrease in tumor growth by NDRG1/Cap43 overexpression in a HCC cell line, KIM-1, in vivo

We subsequently examined whether the overexpression of NDRG1/Cap43 could modulate tumor growth in mice under xenograft assay systems. The tumor growth of KIM-1/Cap43 showed markedly reduced rates as compared with its mock-transfected lines, KIM-1/Mock and (Fig. 4A). H&E analysis showed no apparent morphological differences in tumor samples 7 weeks after inoculation between cell lines with and without NDRG1/Cap43 (Fig. 4B). NDRG1/Cap43 expression in cancer cells was confirmed immunohistochemically in xenografts of KIM-1/Cap43 and HAK-1B/Cap43 cells (Fig. 4B). By contrast, there was almost no expression of NDRG1/Cap43 in xenografts of KIM-1/Mock and HAK-1B/Cap43. We next compared G_0/G_1-specific cell cycle related markers, p21 and CDK4, and also a representative proliferation marker, Ki-67 in mouse xenografts by immunohistochemical analysis. The rate of p21 positive cells in KIM-1/Cap43 tumor was significantly ($P < 0.05$) higher than in those of KIM-1/Mock tumor (Fig. 4C). On the other hand, the rate of CDK4 positive cells in xenograft of KIM-1/Cap43 was significantly ($P < 0.05$) lower than in that of KIM-1/Mock. The Ki-67 labeling index was significantly lower in KIM-1/Cap43 tumor than in the control KIM-1/Mock tumor (Fig. 4C). We did not observe any apparent difference in tumor microvascular density between KIM-1/Mock and KIM-1/Cap43 tumor (data not shown).

3.4. Tumor growth suppression by NDRG1/Cap43 overexpression in another HCC cell line, HAK-1B

To further confirm the suppressive effect of NDRG1/Cap43 overexpression on tumor growth by HCC cells *in vivo*, we established NDRG1/Cap43 overexpressing cell line from a different HCC cell line. Of two HCC cell lines, HAK-1A and HAK-1B, HAK-1A was derived from well differentiated type HCC and HAK-1B was from poorly differentiated type [22]. NDRG1/Cap43 is also a differentiation regulated protein. HAK-1A showed higher expression of NDRG1/Cap43 than HAK-1B (Fig. 5A). HAK-1B/Cap43 that was established by transfection of NDRG1/Cap43 cDNA into HAK-1B expressed a much higher expression of NDRG1/Cap43 than its parental counterpart HAK-1B/Mock (Fig. 5B). Western blot analysis demonstrated an increase in p21 and a decrease in CDK4 in HAK-1B/Cap43 cells in culture under full confluent condition as compared with its control counterpart (Fig. 5C). Tumor growth by HAK-1B/Cap43 demonstrated marked reduction as compared with HAK-1B/Mock (Fig. 5D). In a different HCC cell line, NDRG1/Cap43 overexpression thus induced a suppressive effect on the tumor growth.

4. Discussion

In our present study, we established hepatic cancer KIM-1/Cap43 cell line expressing stably higher amounts of NDRG1/Cap43, and observed the following characteristics. (1) The growth rate of KIM-1/Cap43 was significantly slower *in vitro* compared with that of the control KIM-1/Mock, and tumor growth in the xenograft model system was markedly suppressed by NDGR1/Cap43 overexpression; (2) Expression of G_0/G_1-specific p21 was up-regulated in confluent culture condition *in vitro* and in the tumor of mouse xenograft by NDRG1/Cap43 overexpression; (3) Treatment with mimosine, an inhibitor of G_1/G_0, also increased the expression of NDRG1/Cap43 together with increased expression of p21 and decreased expression of CDK4; (4) Immunohistochemical analysis showed much less expression of CDK4 in cancer cells of KIM-1/Cap43 tumor than those of KIM-1/Mock tumor, and KIM-1/Mock tumor showed the appearance of more Ki-67-positive cancer cells than KIM-1/Cap43 tumor. Taking these findings together, the marked suppression of tumor growth by NDRG1/Cap43 might be due to the activation or the loss of cell cycle or proliferation-promoting biomarkers, such as p21, CDK4 and Ki-67, respectively. Furthermore, we established another NDRG1/Cap43 overexpressing cell line

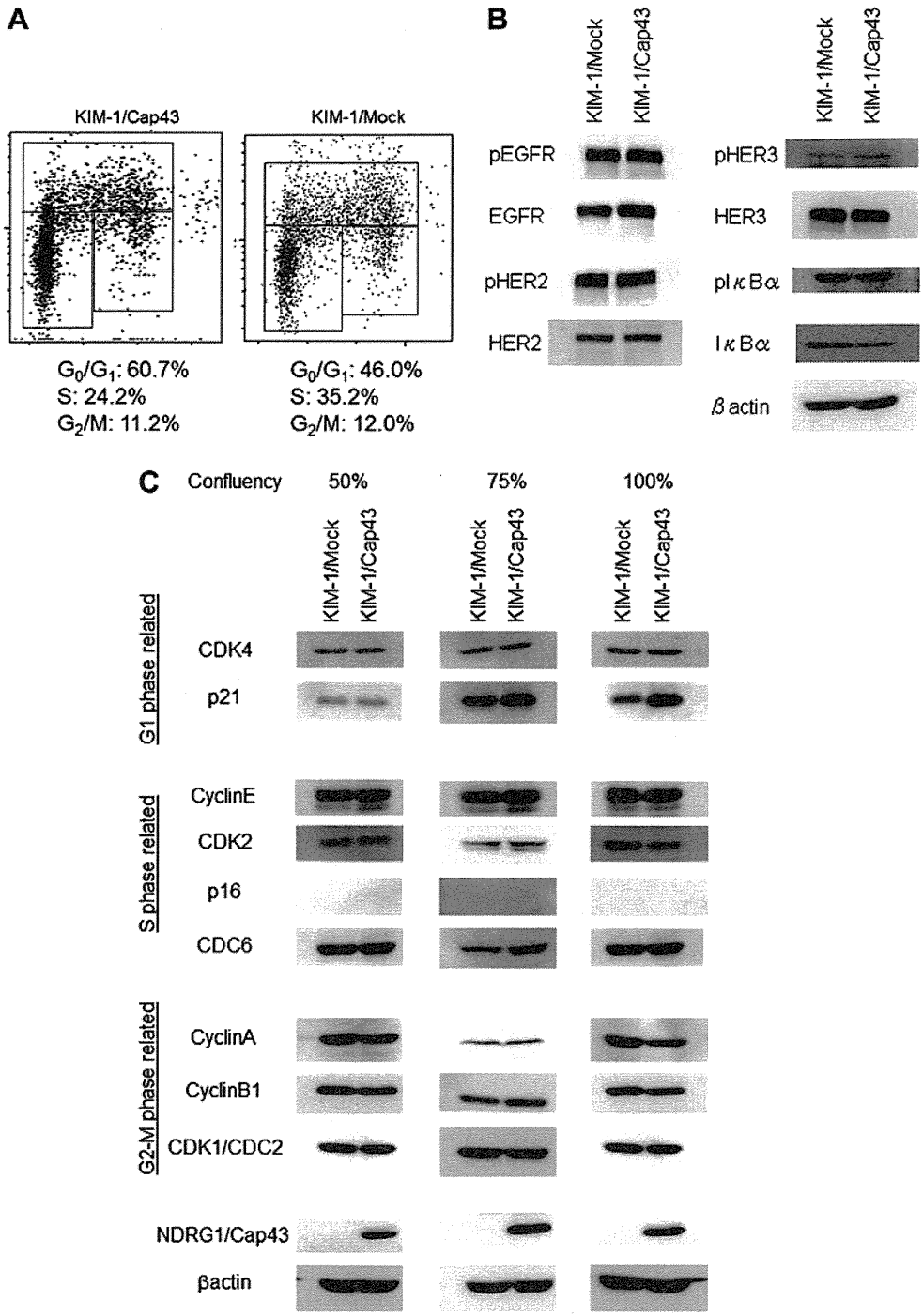

Fig. 2. Effect of NDRG1/Cap43 on cell cycle and cell cycle regulators. (A) KIM-1/Cap43 and KIM-1/Mock were cultured in serum-free culture medium for 24 h. After culturing in medium containing 10% FBS for 12 h, cells were subjected to cell cycle analysis. G_0/G_1 phase cell cycle arrest was observed in NDRG1/Cap43 transfectant, KIM-1/Cap43, as compared with mock transfectant, KIM-1/Mock. (B) There was no apparent difference in various growth factor receptors and their phosphorylated proteins. Expression of IκBα and pIκBα was reduced in KIM-1/Cap43 as compared to KIM-1/Mock. (C) Expression of several cell cycle regulators was examined. Induction of p21 was observed in KIM-1/Cap43 under confluent culture condition. The other cell cycle regulators were not different despite NDRG1/Cap43 expression.

using HAK-1B. A stable transfectant of NDRG1/Cap43, HAK-1B/Cap43 showed p21 induction accompanied by CDK4 reduction in confluent culture condition *in vitro*. NDRG1/Cap43 overexpression also resulted in suppression of tumor growth of HAK-1B, similar to the suppressive effect in KIM-1. Considering these findings together, we favor the idea that NDRG1/Cap43 functions as tumor growth suppression in HCC.