Clinical characteristics of hepatocellular carcinoma in elderly patients

TAKUYA HONDA, HISAMITSU MIYAAKI, TATSUKI ICHIKAWA, NAOTA TAURA, SATOSHI MIUMA, HIDETAKA SHIBATA, HAJIME ISOMOTO, FUMINAO TAKESHIMA and KAZUHIKO NAKAO

Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8501, Japan

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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

Correspondence to: Dr Hisamitsu Miyaaki, Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
E-mail: miyaaki-hi@umin.ac.jp

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.

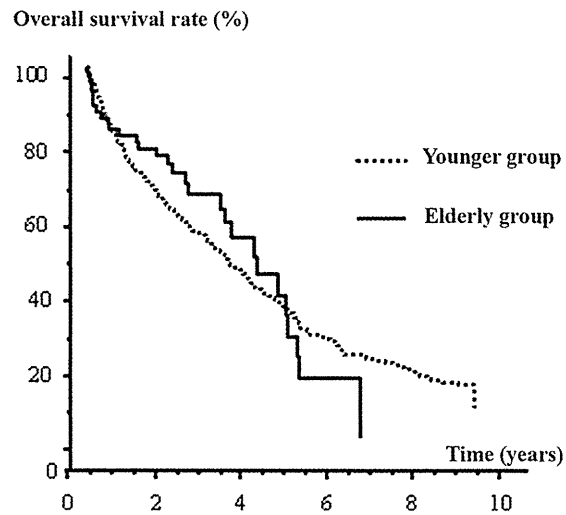

Figure 1. Kaplan-Meier model of the overall survival rate for the younger and elderly groups (younger HCC, 544 cases; elderly HCC, 80 cases). The overall survival between the younger and elderly HCC groups was not significantly different (p=0.665).

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.

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Original Article

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

Hisamitsu Miyaaki,[1] Tatsuki Ichikawa,[1] Hiroshi Yatsuhashi,[3] Naota Taura,[1] Satoshi Miuma,[1] Tetsuya Usui,[2] Sayaka Mori,[2] Shimeru Kamihira,[2] Yasuhito Tanaka,[4] Masashi Mizokami[5] and Kazuhiko Nakao[1]

[1]*Department of Gastroenterology and Hepatology, Nagasaki University School of Medicine, and* [2]*Central Diagnostic Laboratory of Nagasaki University Hospital, Nagasaki, and* [3]*Clinical Research Center, National Hospital Organization (NHO) Nagasaki Medical Center, Omura, and* [4]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, and* [5]*Research Center for Hepatitis and Immunology, International Medical Center of Japan, Konodai Hospital, Ichikawa, Japan*

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

Correspondence: Dr Hisamitsu Miyaaki, Department of Gastroenterology and Hepatology, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Email: miyaaki-hi@umin.ac.jp

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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

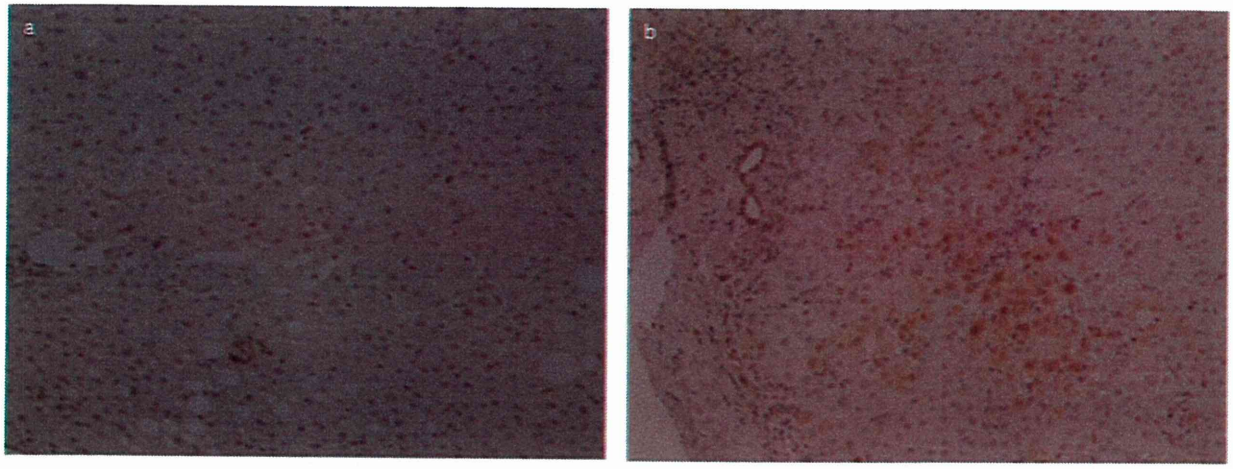

Figure 1 (a) This case showed less than 5% suppressor of cytokine signaling 3 (SOCS3) immunostained areas (low immunostaining). (b) This cases showed about 50% SOCS3 immunostaining areas (high immunostaining).

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

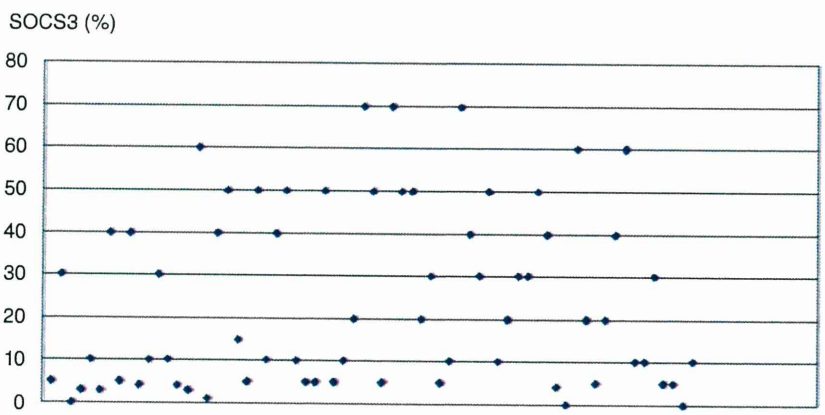

Figure 2 The distribution of the SOCS3 immunostaining area is shown.

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.

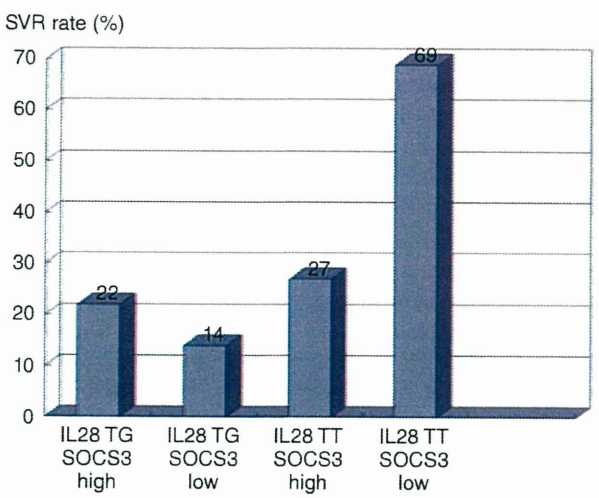

Figure 3 A total of 12.5% of patients with IL28 TG and suppressor of cytokine signaling 3 (SOCS3) high immunostaining showed a sustained virological response (SVR), 20% of patients with IL28 TG and SOCS3 low immunostaining, 31% of patients with IL28 TT and SOCS3 high immunostaining, and 68% of patients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

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Clinical Characteristics of Seven Patients with *Aeromonas* Septicemia in a Japanese Hospital

Yoshitomo Morinaga,[1] Katsunori Yanagihara,[1] Nobuko Araki,[1] Yosuke Harada,[1,2] Koichi Yamada,[1,2] Norihiko Akamatsu,[1] Junichi Matsuda,[1] Tomoya Nishino,[2] Hiroo Hasegawa,[1] Koichi Izumikawa,[2] Hiroshi Kakeya,[2] Yoshihiro Yamamoto,[2] Akira Yasuoka,[3] Shigeru Kohno[2,4] and Shimeru Kamihira[1]

[1]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
[2]Second Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
[3]Nagasaki University Infection Control and Education Center, Nagasaki University Hospital, Nagasaki, Japan
[4]Global COE Program, Nagasaki University, Nagasaki, Japan

The genus *Aeromonas* comprises flagellated gram-negative rods widely distributed in freshwater, estuarine and marine environments. *Aeromonas* species may cause a variety of illnesses in humans, such as enterocolitis and septicemia, especially in warmer tropical or subtropical environments. To recognize the characteristics of *Aeromonas* septicemia in Japan, we reviewed laboratory data and medical records in our hospital. During 11 years (from 2000 to 2010), *Aeromonas* septicemia was observed in seven patients involving six female subjects. Six patients were observed in summer or fall. The incidence of *Aeromonas* septicemia was about 0.07 per 1000 admissions, and two out of the seven patients died. All patients had underlying diseases such as malignancy (six patients) and choledocholithiasis (one patient). Two patients developed septicemia within two days after ingesting raw seafood. Five patients developed *Aeromonas* septicemia > 48 h after admission. Fever was present in all patients, and four out of the seven patients developed septic shock. All patients developed monomicrobial septicemia. *A. hydrophila* was isolated from five patients, and *A. caviae* and *A. veronii* biovar *sobria* were isolated from one patient each. Most antimicrobial agents had high activity against the isolated strains. However, a carbapenem-resistant strain appeared in one patient during treatment and led to death. *Aeromonas* septicemia is uncommon in temperate areas but can occur particularly in warm seasons. Immunocompromised conditions and recent ingestion of raw fish or shellfish are important characteristics of developing *Aeromonas* septicemia.

Keywords: *Aeromonas caviae*; *Aeromonas hydrophila*; *Aeromonas veronii* biovar *sobria*; *Aeromonas* septicemia; seafood
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The genus *Aeromonas* comprises flagellated gram-negative rods widely distributed in freshwater, estuarine and marine environments. Although *Aeromonas* species can grow at a range of temperatures, they are isolated more frequently in warmer tropical or subtropical environments. They have the potential to infect or colonize humans and are associated with a variety of illnesses, such as enterocolitis (Holmberg and Farmer 1984), septicemia (Janda et al. 1994), skin and soft tissue infections (Furusu et al. 1997), and peritonitis (Huang et al. 2006).

Severe infections sometimes occur in clinically ill patients and septicemia is an important invasive disease associated with *Aeromonas* infections. *Aeromonas* infections can develop into septicemia not only in immunocompromised patients but in trauma patients as well as healthy persons. However, immunocompromised patients with hepatobiliary diseases or hematologic malignancy are considered to be at the greatest risk for *Aeromonas* septicemia (Tsai et al. 2006).

Some studies with large numbers of patients with *Aeromonas* infections have been reported from countries located in tropical or subtropical climates (Ko et al. 2000; Lau et al. 2000). However, epidemiological differences in *Aeromonas* infections have been noted to be based on geographic locales or populations (Janda and Abbott 2010). Although increasing antibiotic resistance in clinical isolates has been reported in Taiwan (Ko et al. 1996), the patterns of susceptibility to drugs may also vary due to geographic

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Correspondence: Katsunori Yanagihara, M.D., Ph.D., Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
e-mail: k-yanagi@nagasaki-u.ac.jp

locations or selection pressures.

Because Japan lies in a temperate zone and has four distinct seasons, the characteristics of *Aeromonas* infections in Japan are different from those in tropical or subtropical countries, and *Aeromonas* septicemia is relatively uncommon in Japan. To understand the characteristics of patients with *Aeromonas* septicemia in Japan, we retrospectively reviewed patients at our hospital.

Materials and Methods

Nagasaki University Hospital is a facility with about 850 beds. Hospital microbiology laboratory databases were reviewed for all species of *Aeromonas* isolated between the years 2000 to 2010. Positive blood culture for *Aeromonas* was considered to represent evidence of septicemia. Primary septicemia was defined in patients with fever or hypotension without an apparent focus or portal of entry. Data on clinical characteristics were obtained retrospectively by reviewing patient medical records. The laboratory parameters were extracted from same- or next-day blood sampling data. Patients were identified as either community-acquired or healthcare-associated. Healthcare-associated patients were defined as having occurred > 48 h after hospital admission, with signs and symptoms of infection that were absent at the time of admission. *Aeromonas* species were isolated on blood agar and identified to the species level by using the Vitek2 system (bioMerieux, Hazelwood, MO, USA) or Phoenix 100 instrument (BD, Franklin Lakes, NJ, USA). Antimicrobial susceptibility testing of *Aeromonas* isolates was performed by the hospital microbiology laboratory by broth microdilution assay according to the Clinical and Laboratory Standards Institute (The Clinical and Laboratory Standards Institute 2010).

Results

From the years 2000 to 2010, *Aeromonas* species were isolated from 101 patients at Nagasaki University Hospital. *Aeromonas* species were isolated from the blood samples of seven patients (6.9%) (Table 1). The incidence of *Aeromonas* septicemia in our hospital was about 0.07 per 1,000 admissions. The patient subjects included one male and six females, ranging in age from 15 to 89 years (median age, 76 years). *A. hydrophila* was isolated from five patients, and *A. caviae* and *A. veronii* biovar *sobria* were isolated from one patient each. Malignant diseases were observed in six patients (85.7%). Four patients had hematological malignancies, one had colon cancer, and one had pancreatic cancer. None of the patients had liver cirrhosis as an underlying condition. Two patients presented secondary septicemia followed by cholangitis associated with stenosis due to choledocholithiasis or pancreatic cancer. The other patients presented primary septicemia. In two patients primary septicemia developed during nadir of neutropenia due to chemotherapy.

One hundred percent of the patients had fevers and four patients (57.1%) developed septic shock. Five (71.4%) of the seven patients were healthcare-associated. As episodes, two patients (patients 1 and 2) had ingested raw seafood within 2 days prior to developing symptoms. Patient 1 consumed raw sea bream and manila clam at home. Patient

Table 1. Clinical summary of seven patients with *Aeromonas* septicemia.

Patient no.	Age	Sex	Onset month	Isolates	Comorbidities	Clinical presentation	Day of septicemia onset after hospitalization	Body temperature (°C)	Abdominal pain	Shock	WBC (/μL)	Drugs	Outcome
1	74	F	Jul	*A. hydrophila*	Choledocholithiasis	Cholangitis + septicemia	(community-acquired)	39.8	+	−	12,400	Meropenem	Cured
2	35	F	Jul	*A. hydrophila*	Colon cancer	Primary septicemia	17	39.9	−	−	5,900	Biapenem	Cured
3	79	F	Jul	*A. hydrophila*	Multiple myeloma, Diabetes	Primary septicemia	169	38.3	+	−	2,300	None	Died
4	76	F	Sep	*A. hydrophila*	MDS/ALL	Primary septicemia	(community-acquired)	39.2	−	+	13,400	Cefepime + Isepamicin	Cured
5	78	M	Feb	*A. caviae*	AML	Primary septicemia	26	39.5	−	+	100	Meropenem	Cured
6	89	F	Oct	*A. hydrophila*	Pancreatic cancer	Cholangitis + septicemia	9	40.1	−	+	9,100	Imipenem	Cured
7	15	F	Jul	*A. veronii* biovar *sobria*	ALL, Down syndrome	Primary septicemia	371	40.4	−	+	100	Meropenem	Died

WBC, white blood cell; MDS, myelodysplastic syndromes; ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia.

2 had a history of eating unspecified raw fish that were brought by family during hospitalization. In addition, the stool of patient 2 was cultured, but *Aeromonas* species were not isolated.

With antimicrobial therapy, five out of six patients received a carbapenem as a first-line therapy. Patient 1 required endoscopic sphincterotomy to remove a common bile duct stone. Ceftazidime, cefepime, aztreonam, meropenem, gentamicin, and ciprofloxacin showed good antimicrobial activity against *Aeromonas* species isolated in the study, although ampicillin/sulbactam did not.

Two patients (28.6%) died due to *Aeromonas* septicemia. For patient 3, aggressive treatment with antibiotics was not selected because the patient was in a terminal state of multiple myeloma. For patient 7, meropenem was started for the treatment of neutropenic fever. However, the effect of treatment with meropenem alone was insufficient. Combination treatment with meropenem, vancomycin, and ciprofloxacin was then selected. The minimum inhibitory concentration (MIC) of meropenem against the strain isolated at onset was ≤ 0.5 μg/mL, but ≥ 32 μg/mL on the seventh day after septicemia onset. The MIC of ciprofloxacin remained at ≤ 0.5 μg/mL in each isolate. However, the patient's condition did not respond to the treatment, resulting in death on the tenth day after onset.

Discussion

Aeromonas species are universally isolated from a variety of environmental sources, including water, seafood, meats and vegetables (Palumbo et al. 1985). Their growth is encouraged in thermal gradients ranging from 25°C to 35°C (Hazen et al. 1978) and *Aeromonas* infections can be relatively frequent in warm tropical or subtropical climates. Therefore, many reports concerning *Aeromonas* infections have been published from these areas (Chan et al. 2000; Ko et al. 2000; Tsai et al. 2006). Indeed, the number of patients with *Aeromonas* septicemia in our hospital during the past 11 years was small compared to reports from Taiwan (Ko et al. 2000; Lau et al. 2000). Characteristics of the Japanese climate may affect bacterial growth, especially in winter. Japanese people may also have fewer opportunities to be exposed contaminated water or foods containing high concentrations of bacteria compared to people in subtropical countries. In fact, six out of seven of patients were observed between July and September, the period when the sea temperature is relatively warm.

A. hydrophila, *A. caviae,* and *A. veronii* biovar *sobria* are major *Aeromonas* species that cause septicemia (Janda et al. 1994). These species were also observed in our study. In several reports of *Aeromonas* septicemia, the male-female ratio ranged from 1.6 to 4.0 and community-acquired patients comprised 71 to 79% of all patients (Ko et al. 2000; Lau et al. 2000; Llopis et al. 2004). In contrast, our study involved a lower percentage of men and a lower percentage of community-acquired patients. The difference in our male-female ratio may be due to the few patients in our study. In regards to the lower percentage of community-acquired patients, fewer opportunities of contact with *Aeromonas* species may decrease the potential of septicemia. In contrast, *Aeromonas* septicemia was observed mainly in hospitalized patients. The fact that *Aeromonas* strains colonize in multiple ways and the host develops a serious immunosuppressive condition requiring hospitalization may increase the potential of bacteremia. However, we should also pay attention to food-borne patients from the ingestion of raw fish or shellfish, as in patients 1 and 2. The seafood can carry a lot of *Aeromonas* strains even in Japanese climate, especially in the warm seasons. These results suggest that consumption of *Aeromonas*-contaminated foods such as raw seafood as well as immunocompromised conditions can be risk factors for *Aeromonas* septicemia in temperate areas. Considering that most of the patients presented in this study developed *Aeromonas* septicemia in summer or fall, the patients who had uncertain episodes might also consume some *Aeromonas*-contaminated foods.

Aeromonas septicemia can be categorized into four groups according to the patient's condition: immunocompromised patients, trauma patients, healthy persons, and post-reconstructive surgery patients. *Aeromonas* septicemia are seen mainly in immunocompromised patients (Janda and Abbott 2010). Several studies with a large number of patients reported that predisposing conditions for *Aeromonas* septicemia were chronic liver disease, malignancy, and biliary tract disease (Ko et al. 2000; Lau et al. 2000; Llopis et al. 2004). In individuals with hematologic malignancies, anti-neoplastic medications may cause disintegration of the gastrointestinal mucosa and allow transmigration of colonized *Aeromonas* species from the bowel into the circulatory system. Among hematological malignant diseases, acute myeloblastic leukemia, acute lymphoblastic leukemia, and non-Hodgkin's lymphoma were three leading risk conditions for *Aeromonas* septicemia (Tsai et al. 2006). As reported in these studies, the comorbidities of our patients were similar, except we had no patients with chronic liver disease. Patients whose onset was during nadir of neutropenia or a terminal state had risks of developing bacteremic translocation.

In reports with a large number of patients, the most common symptoms associated with *Aeromonas* septicemia were fever (74 to 89%), jaundice (57%), abdominal pain (16 to 45%) and septic shock (40 to 45%) (Lau et al. 2000; Tsai et al. 2006). Similarly, these symptoms were observed in our patients except for jaundice. Because these symptoms are nonspecific, it is difficult to distinguish *Aeromonas* septicemia from those caused by other gram-negative septicemia by clinical features. The percentage of monomicrobic septicemia (100%) in our study was higher than that in previous reports (64 to 75.6%) (Lau et al. 2000; Llopis et al. 2004; Tsai et al. 2006). Our results may have been influenced by the presence of a few patients with hepatobiliary diseases. When polymicrobic septicemia occurs, *Escheri-*

chia coli, *Klebsiella pneumoniae* and *Staphylococcus aureus* can be identified with *Aeromonas* species (Lau et al. 2000; Tsai et al. 2006). The mortality rate in this study was similar to the result of some previous reports, (Ko et al. 2000; Tsai et al. 2006). However, only in hospitalized patients, the mortality rate in our study was 40%, whereas Ko et al. (2000) reported 15%. The patients who required long-term hospitalization because of their poor conditions seemed to elevate the mortality rate, suggesting that *Aeromonas* septicemia can be fatal in immunocompromised patients.

In conclusion, we reported seven patients of *Aeromonas* septicemia in a university hospital in Japan. Immunocompromised conditions, such as hematological malignancy or cholangitis, and recent ingestion of raw fish or shellfish were important characteristics of developing *Aeromonas* septicemia. All patients were monomicrobial. Most of the isolates were susceptible to antimicrobial agents. However, the appearance of a drug-resistant isolate during the treatment indicates the need for caution in the selection of drugs.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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RESEARCH ARTICLE

The glycosylphosphatidylinositol-linked aspartyl protease Yps1 is transcriptionally regulated by the calcineurin-Crz1 and Slt2 MAPK pathways in Candida glabrata

Taiga Miyazaki[1], Koichi Izumikawa[1], Shunsuke Yamauchi[1], Tatsuo Inamine[2], Yohsuke Nagayoshi[1], Tomomi Saijo[1], Masafumi Seki[1], Hiroshi Kakeya[1], Yoshihiro Yamamoto[1], Katsunori Yanagihara[1], Yoshitsugu Miyazaki[3], Akira Yasuoka[4] & Shigeru Kohno[1]

[1]Department of Molecular Microbiology and Immunology, Nagasaki University School of Medicine, Nagasaki, Japan; [2]Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [3]Department of Bioactive Molecules, National Institutes of Infectious Diseases, Tokyo, Japan; and [4]Infection Control and Education Center, Nagasaki University Hospital, Nagasaki, Japan

Correspondence: Taiga Miyazaki, Department of Molecular Microbiology and Immunology, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Tel.: +81 95 819 7273; fax: +81 95 849 7285; e-mail: taiga-m@nagasaki-u.ac.jp

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Abstract

In the pathogenic fungus *Candida glabrata*, the *YPS1* gene, which encodes a glycosylphosphatidylinositol-linked aspartyl protease, is required for cell wall integrity and virulence. Although the expression of *YPS1* has been studied in *Saccharomyces cerevisiae*, the transcriptional regulation of this gene in *C. glabrata* is not well understood. Here, we report that *C. glabrata* Yps1 is required for cell growth at elevated temperatures, and that the heat-induced expression of *YPS1* is regulated predominantly by the calcineurin-Crz1 pathway and partially by the Slt2 MAPK pathway. Although a total of 11 *YPS* genes are present in the *C. glabrata* genome, the loss of transcriptional induction in a calcineurin mutant was observed only for *YPS1*. The results of a *YPS1* promoter-*lacZ* reporter assay using a series of constructs with mutated promoter elements indicated that the transcription factor Crz1 binds to multiple sites in the promoter region of *YPS1*. To date, as none of the putative Crz1 targets in *C. glabrata* have been characterized using a Δ*crz1* mutant, monitoring the expression of *YPS1* represents an effective method for measuring the activity of the calcineurin-Crz1 signaling pathway in this fungus.

Introduction

In *Saccharomyces cerevisiae*, a family of five glycosylphosphatidylinositol-linked aspartyl proteases (Yps1-3, Yps6, and Yps7), known as yapsins, are required for maintaining cell wall integrity (Krysan *et al.*, 2005; Gagnon-Arsenault *et al.*, 2006). Yapsins have also been identified in the opportunistic fungal pathogen *Candida glabrata*, which contains 11 *YPS* genes (*YPS1*, *YPS2*, *YPS7*, and a cluster of eight *YPS* genes), encoding Yps proteases that play an important role in cell wall remodeling by removing glycosylphosphatidylinositol-anchored cell wall proteins, such as the adhesin Epa1, from the cell wall (Kaur *et al.*, 2007). Kaur *et al.* (2007) have also demonstrated that among the 11 *YPS* genes, *YPS1* plays a primary role in the survival of *C. glabrata* within macrophages and virulence in a mouse model of disseminated candidiasis. Although the expression of *S. cerevisiae YPS1* is induced in association with active cell wall synthesis or remodeling under cell wall-damaging conditions, and is coregulated by the Pkc1-Mpk1/Slt2 and calcineurin-signaling pathways (Krysan *et al.*, 2005; Gagnon-Arsenault *et al.*, 2006), it is not known whether these regulation mechanisms are active in *C. glabrata*. In this study, we therefore attempted to determine whether the Slt2 pathway and calcineurin are involved in the transcriptional regulation of *YPS1* in *C. glabrata*.

Materials and methods

Strains and culture conditions

The *C. glabrata* strains used in this study are listed in Table 1. *Candida glabrata* cells were routinely propagated at 30 °C in synthetic complete medium (SC) or SC lacking uracil (SC-ura) or tryptophan (Sc-trp) to maintain plasmid selection (Kaiser *et al.*, 1994).

Table 1. *Candida glabrata* strains used in this study

Strain	Genotype or description	Reference or source
CBS138	Wild type	Dujon *et al.* (2004)
2001T	Δ*trp1*	Kitada *et al.* (1995)
2001HT	Δ*his3*, Δ*trp1*	Kitada *et al.* (1995)
2001TU	Δ*trp1*, Δ*ura3*	Kitada *et al.* (1995)
TG11	2001T containing pCgACT-P	Miyazaki *et al.* (2010a)
TG151	Δ*slt2*::*HIS3*, Δ*trp1* (made from 2001HT)	Miyazaki *et al.* (2010a)
TG152	TG151 containing pCgACT-P	Miyazaki *et al.* (2010a)
TG153	TG151 containing pCgACT-PS2	Miyazaki *et al.* (2010a)
TG161	Δ*cnb1*::*HIS3*, Δ*trp1* (made from 2001HT)	Miyazaki *et al.* (2010b)
TG162	TG161 containing pCgACT-P	Miyazaki *et al.* (2010b)
TG163	TG161 containing pCgACT-PNB	Miyazaki *et al.* (2010b)
TG164	TG161 containing pCgACT-PY1	This study
TG171	Δ*crz1*::*HIS3*, Δ*trp1* (made from 2001HT)	Miyazaki *et al.* (2010b)
TG172	TG171 containing pCgACT-P	Miyazaki *et al.* (2010b)
TG173	TG171 containing pCgACT-PRZ	Miyazaki *et al.* (2010b)
TG174	Δ*crz1*::*HIS3*, Δ*trp1*, Δ*ura3*	This study
TG181	Δ*rlm1*::*HIS3*, Δ*trp1* (made from 2001HT)	Miyazaki *et al.* (2010a)
TG182	TG181 containing pCgACT-P	Miyazaki *et al.* (2010a)
TG191	Δ*yps1*::*HIS3*, Δ*trp1* (made from 2001HT)	This study
TG192	TG191 containing pCgACT-P	This study
TG193	TG191 containing pCgACT-PY1	This study
TU-EM14	2001TU containing pEM14	This study
TU-Y1	2001TU containing pPYPS1	This study
TU-YD1	2001TU containing pPYPS1-D1	This study
TU-YD2	2001TU containing pPYPS1-D2	This study
TU-YD3	2001TU containing pPYPS1-D3	This study
TU-YD4	2001TU containing pPYPS1-D4	This study
TU-YD42	2001TU containing pPYPS1-D42	This study
TU-YD5	2001TU containing pPYPS1-D5	This study
TU-YD5-4	2001TU containing pPYPS1-D5-4	This study
TU-YD52	2001TU containing pPYPS1-D52	This study
TU-YD6	2001TU containing pPYPS1-D6	This study
TG174-Y1	TG174 containing pPYPS1	This study

Deletion and complementation of YPS1 in C. glabrata

Sequence information of *C. glabrata* genes was obtained from the *C. glabrata* genome database Genolevures (http://www.genolevures.org/). The primers and plasmids used in this study are listed in Tables 2 and 3, respectively. The plasmids constructed using PCR products were verified by sequencing before use. Transformation of *C. glabrata* was performed using a lithium acetate protocol, as described previously (Cormack & Falkow, 1999).

A *C. glabrata YPS1* deletion strain was generated using a one-step PCR–based technique, as described previously (Miyazaki *et al.*, 2010a). Briefly, a deletion construct was amplified from pBSK-HIS (Miyazaki *et al.*, 2010a) with the primer pair CgYPS1 100-F and CgYPS1 100-R. *Candida glabrata* strain 2001HT (Δ*his3*, Δ*trp1*) (Kitada *et al.*, 1995) was then transformed with the deletion construct, and the resulting transformants were selected by histidine prototrophy. Both PCR and Southern blotting were performed to verify that the desired homologous recombination occurred at the target locus without ectopic integration of the deletion construct. One successful deletion strain (Δ*yps1*) was selected and designated as TG191.

To generate a complementation plasmid, a 1836-bp DNA fragment containing the *C. glabrata YPS1* gene was amplified from the genomic DNA of the *C. glabrata* wild-type strain CBS138 (Dujon *et al.*, 2004) with the primer pair CgYPS1-Xba-F and CgYPS1-Xba-R. The PCR product was digested with XbaI and inserted into the XbaI site of pCgACT-P to generate pCgACT-PY1. Strain TG191 was then transformed with pCgACT-P and pCgACT-PY1 to generate strains TG192 and TG193, respectively.

Spot dilution test

To examine sensitivity to high temperature, a spot dilution test was performed as described previously (Miyazaki *et al.*, 2010a). Briefly, logarithmic-phase cells grown in SC-trp broth were harvested and adjusted to 2×10^7 cells mL^{-1}. Serial 10-fold dilutions were then prepared, and 5 μL of each dilution was spotted onto SC-trp agar plates in the presence and absence of 1 M sorbitol. Plates were incubated for 48 h at 30 or 41 °C. The spot dilution test was repeated twice on independent occasions.

Quantitative real-time reverse transcription (RT)-PCR

To examine the expression levels of the *YPS* genes, logarithmic-phase cells grown in SC-trp broth at 30 °C were adjusted to 1×10^7 cells mL^{-1} and then further incubated at 41 °C with agitation. Total RNA was extracted using a FastRNA Red Kit (Qbiogene, Carlsbad, CA). Quantitative real-time RT-PCR was performed as described previously (Saijo *et al.*, 2010). Briefly, first-strand cDNA was synthesized using a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) from 1 μg of total RNA in a final volume of 20 μL, and 3 μL of resulting cDNA was then used as a template for individual PCR with gene-specific primers (Table 2), using a QuantiTect SYBR Green PCR kit (Qiagen). Quantitative real-time RT-PCR was performed in triplicate in a 96-well plate format, using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The mRNA abundance of the *YPS* genes was normalized to *ACT1* transcript levels. The real-time RT-PCR assays were repeated twice on independent occasions.

Table 2. Primers used in this study

Primer*	Sequence (5′–3′)†
For strain and plasmid construction	
CgYPS1 100-F	*CACGCTAATGGCTTGGGGGGGAAGGGCAAGGGCATTAACTGCTGATGGGG CTCCCCTGCAAGGCTTGGATCTTG ATAATTGCCATATATATAGCTTGTA***TAATACGACTCACTATAGGGC**
CgYPS1 100-R	*GCATTTAAAGAACTCCAGCTTTGCTTAATATCAAATGAACAAAGA AATCCTCACCAGGAGCACCGGTTGATG CTATTCCAGGGTGAGAGCCATCTTCAG*CGCTCTAGAACTAGTGGATCC
CgYPS1-Xba-F	GCTCTAGAATGAAGTTTAGTTCGCTATGTATGC
CgYPS1-Xba-R	GCTCTAGAGGGTGAGAGCCATCTTCAGAATG
CgYPS1-355Bam-F	CGGGATCCTGAAGCCCGAGAGAAATCCC
CgYPS1-38Kpn-R	AAGGTACCGCAACAGACGCCAGCATACATAG
YPS1prm-F1	TTGCGCATCTTGAAAAAACAA
YPS1prm-F2	CCCTGCAAGGCTTGGATCT
YPS1prm-F3	CTTGTAAAATGTGATTCCTGAATACC
YPS1prm-F4	TTTCTTTTTCACGCTAATGGC
YPS1prm-F5	TGGGGGGGAAGGGCAAGG
YPS1prm-F6	TGGATCTTGATAATTGCCA
YPS1prm-R1	GTGCTTTGCTTCTTTGCTTC
YPS1prm-R2	ATCAGCAGTTAATGCCCTTGC
YPS1prm-R3	GGCAATTATCAAGATCCAAGC
YPS1prm-R4	GCTTTAATTTGAATTTTCTTC
YPS1prm-R5	TTTTCAAGCCCTTTTCAAGCCCTTT
For real-time PCR	
CgYPS1-F1281	CGGATTCCACATCAACGCTC
CgYPS1-R1436	TCGTAGTTTTCCAGGTCGTAGACG
CgYPS2-F1518	CACTGAGTTCCCATCTTCTTATGCC
CgYPS2-R1647	CGTGTTTGTGTTTCTGCTTGTCC
CgYPS3-F944	ACGCACCAAAGCAAGTCGTC
CgYPS3-R1079	GCACCAGCAAAGTTGAAGATAAGC
CgYPS4-F490	AATGGCGTGAAGGTTGATAACG
CgYPS4-R603	GGCAGCAAGTTGATTTGTTGTCTC
CgYPS5-F312	TGATGGATTCCCCACTTCGC
CgYPS5-R461	GCAAATGTCTCGTCACCGTAGC
CgYPS6-F305	CTGGCTTTCCAACAAACCCTG
CgYPS6-R495	CAATCCACCCAAACTAACCACATC
CgYPS7-F438	CAGCGGTGTGAGTTTCAGGAAC
CgYPS7-R602	AGGAAGAAGAACGAGTGGTTGATG
CgYPS8-F510	TGTGAACTTTGCCTTGGGGAG
CgYPS8-R632	GAATCGCTGATGTTTTTCCTGC
CgYPS9-F70	AAGACCCCAGGCAGTGATTTG
CgYPS9-R198	TTGTAGCAAGACCTTCTGAGGAGG
CgYPS10-F310	GCACAGGTTCCCAAATCGTTG
CgYPS10-R439	CAGCGTAGGATGAGTCCAAGTAGC
CgYPS11-F1269	TGACACTTTGCCAGAAGACATCG
CgYPS11-R1414	TTGTGCTGCCGTTTGAGGAG
CgACT1-F163	GGTATGGGTCAAAAGGACTCTTACG
CgACT1-R305	TCGTTGTAGAAAGTGTGATGCCAG

*'F' and 'R' indicate forward and reverse primers, respectively.
†Sequences homologous to flanking regions of the *YPS1* ORF are shown in italics. Sequences shown in bold are present in pBSK-HIS. Restriction sites are underlined.

Construction of lacZ-reporter plasmids

A 0.4-kb fragment containing the 355-bp upstream region and the first 12 codons of *C. glabrata YPS1* was amplified from the genomic DNA of *C. glabrata* CBS138 with the primers CgYPS1-355Bam-F and CgYPS1-38Kpn-R. The resulting PCR product was digested with BamHI and KpnI and inserted into the corresponding sites of pEM14 (El Barkani *et al.*, 2000) to yield pPYPS1. Various deletions were introduced into the *YPS1* promoter sequence in pPYPS1

Table 3. Plasmids used in this study*

Plasmid	Description	Reference or source
pBSK-HIS	A 1-kb XhoI fragment containing *C. glabrata HIS3* was inserted into the XhoI site of pBluescript II SK+(Stratagene).	Miyazaki *et al.* (2010a)
pCgACT-P	A 1-kb SacI–KpnI fragment containing the *S. cerevisiae PGK1* promoter, polylinker and *C. glabrata HIS3* 3′UTR was excised from pGRB2.2 and inserted into the SacI-KpnI site of pCgACT.	Miyazaki *et al.* (2010a) Frieman *et al.* (2002) Kitada *et al.* (1996)
pCgACT-PS2	A 1625-bp fragment containing *C. glabrata SLT2* was inserted into the SalI site of pCgACT-P	Miyazaki *et al.* (2010a)
pCgACT-PNB	A 580-bp fragment containing *C. glabrata CNB1* was inserted into the SmaI site of pCgACT-P	Miyazaki *et al.* (2010b)
pCgACT-PRZ	A 1938-bp fragment containing *C. glabrata CRZ1* was inserted into the EcoRI site of pCgACT-P	Miyazaki *et al.* (2010b)
pCgACT-PY1	A 1836-bp fragment containing *C. glabrata YPS1* was inserted into the XbaI site of pCgACT-P	This study
pEM14	*C. glabrata* centromere and autonomously replicating sequence-based plasmid containing *S. cerevisiae URA3* and *E. coli lacZ*	El Barkani *et al.* (2000)
pPYPS1	A 0.4-kb fragment containing the 355-bp promoter region and the first 12 codons of *C. glabrata YPS1* was inserted into the BamHI-KpnI site of pEM14.	This study

*The series of lacZ-reporter plasmids constructed from pPYPS1 are summarized in Fig. 3b.

using the KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan) and mutagenic primers (Table 2). Plasmid pPYPS1-D2 was used as a template to generate plasmids pPYPS1-D42 and pPYPS1-D52. All plasmids generated by site-directed deletion mutagenesis were verified by DNA sequencing.

β-Galactosidase assay

Candida glabrata strain 2001TU (Δ*trp1*, Δ*ura3*) (Kitada *et al.*, 1995) was transformed with the series of constructed *YPS1* promoter-*lacZ* reporter plasmids. Strain TG174 (Δ*crz1*, Δ*trp1*, Δ*ura3*), which was obtained by plating strain TG171 (Δ*crz1*, Δ*trp1*) (Miyazaki *et al.*, 2010b) on 5-fluoro-orotic acid-containing media as described previously (Boeke *et al.*, 1984), was transformed with pPYPS1. Transformants were selected by uracil prototrophy and verified by PCR using plasmid-specific primers. Logarithmic-phase cells grown in SC-ura broth were adjusted to 1×10^7 cells mL^{-1} and subjected to a temperature shift from 30 to 41 °C. After 3 h of incubation at 41 °C, the cell cultures were harvested and washed twice with ice-cold phosphate-buffered saline. Cells (100 μL) were resuspended in 300 μL Reporter Lysis Buffer (Promega, Madison, WI) containing 5 μL Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Cell extracts were prepared using acid-washed glass beads (Sigma) and cleared by centrifugation at 14 000 ***g*** for 30 min at 4 °C. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. β-Galactosidase activity was measured using the β-Galactosidase Enzyme Assay System (Promega) according to the manufacturer's instructions. All assays were performed in triplicate on separate days. β-Galactosidase activities of each reporter construct were calculated in Miller units (nmol min^{-1} mg^{-1} protein) at 37 °C (Miller, 1972). The activity of each mutated promoter is expressed relative to the value obtained with the intact promoter.

Results and discussion

Candida glabrata requires Yps1 for cell growth at elevated temperatures

Several phenotypic differences in the Δ*yps1* strains of *S. cerevisiae* and *C. glabrata* have been reported. For example, while the loss of Yps1 results in decreased tolerance to Congo red and caspofungin in *S. cerevisiae* (Krysan *et al.*, 2005), a similar sensitivity is not displayed by *C. glabrata* (Kaur *et al.*, 2007). As the effect of *YPS1* deletion on temperature sensitivity has not been reported in *C. glabrata*, we examined the growth of the *C. glabrata* Δ*yps1* strain at 30 and 41 °C using a spot dilution assay (Fig. 1). The growth of the mutant was similar to that of the wild-type strain at 30 °C, but it was drastically impaired at 41 °C. However, either reintroduction of the wild-type *YPS1* gene or the addition of 1 M sorbitol, an osmotic stabilizer, to the medium could effectively restore the growth of the mutant. These results suggest that *C. glabrata* Yps1 plays a role in maintaining cell wall integrity to prevent cell lysis at elevated temperatures.

Heat-induced expression of YPS1 is predominantly regulated by the calcineurin-Crz1 pathway in C. glabrata

To examine whether the transcription of *C. glabrata YPS1* is upregulated in response to heat stress, we monitored the expression levels of *YPS1* mRNA using real-time RT-PCR. Because the signaling pathways controlling *YPS1* expression in *C. glabrata* are unclear, we also examined *YPS1* expression levels in mutants lacking a key component of two cell wall-associated signaling pathways, including the last member of the PKC1-MAPK cascade (Slt2), the regulatory B subunit of the serine–threonine-specific protein phosphatase

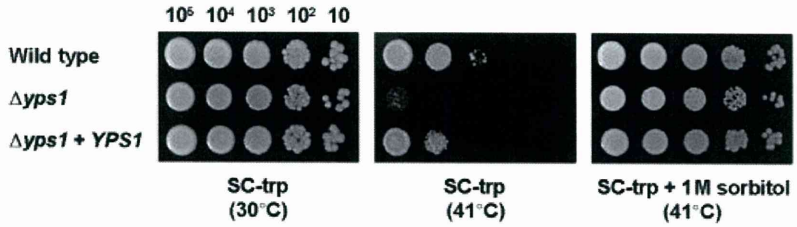

Fig. 1. Yps1 is required for cell growth at an elevated temperature in *Candida glabrata*. After logarithmic-phase cells were adjusted to 2×10^7 cells mL^{-1}, 5 μL of serial 10-fold dilutions were spotted onto an SC-trp agar plate in the presence and absence of 1 M sorbitol. Plates were photographed after 48 h of incubation at the indicated temperatures. Representative results of two independent experiments are shown. *Candida glabrata* strains: wild type, 2001T containing an empty vector (strain TG11); Δ*yps1*, a Δ*yps1* strain containing an empty vector (strain TG192); and Δ*yps1*+*YPS1*, a Δ*yps1* strain containing pCgACT-PY1 (strain TG193).

calcineurin (Cnb1), and the calcineurin-regulated transcription factor Crz1. The expression level of *C. glabrata YPS1* increased in the wild-type strain 1 h after a temperature shift from 30 to 41 °C, and this upregulation was sustained for the duration of the 4-h experiment (Fig. 2a), which is consistent with the kinetics of most genes involved in cell wall remodeling (Garcia *et al.*, 2004). Notably, the transcriptional upregulation of *YPS1* was not observed in the Δ*cnb1* or Δ*crz1* mutants (Fig. 2a). In *S. cerevisiae*, the treatment of wild-type cells with sodium dodecyl sulfate or calcofluor white leads to increased expression of *YPS1*, but has no effect in a strain lacking *MPK1/SLT2* (Krysan *et al.*, 2005; Gagnon-Arsenault *et al.*, 2006). Here, we observed that the deletion of *SLT2* in *C. glabrata* impaired the heat-induced expression of *YPS1* compared with the wild-type strain; however, expression was not completely abolished (Fig. 2a). Rlm1 is a transcription factor downstream of Slt2 and responsible for most of the transcriptional activation of genes required for cell wall integrity in *S. cerevisiae* (Garcia *et al.*, 2004). However, the *YPS1* expression levels in a *C. glabrata* Δ*rlm1* mutant (Miyazaki *et al.*, 2010a) were similar to those in the wild-type strain after 3 h of incubation at 41 °C (data not shown), indicating that Slt2 regulated *YPS1* expression independent of Rlm1 in *C. glabrata*. The results were consistent with previous findings in *S. cerevisiae* that induction of *YPS1* expression after exposure to calcofluor white requires Slt2, but not Rlm1 (Krysan *et al.*, 2005). Further studies investigating how Slt2 regulates *YPS1* expression in these fungi are warranted.

We also compared the heat-induced expression levels of *YPS1* in the *CNB1*-, *CRZ1*-, and *SLT2*-overexpressing strains with that of the wild-type *C. glabrata* strain (Fig. 2b). Although the expression levels of *YPS1* in the *CNB1*- and *SLT2*-overexpressing strains were comparable to that of the wild type, the expression of *YPS1* in the *CRZ1*-overexpressing strain increased approximately fivefold even in the absence of heat stress and increased > 20-fold at 41 °C (Fig. 2b). Taken together, these results suggest that stimulated expression of *YPS1* in *C. glabrata* in response to elevated growth temperature is regulated by both the Slt2 MAPK pathway and calcineurin signaling via the Crz1 transcription factor, with the latter representing the dominant mechanism.

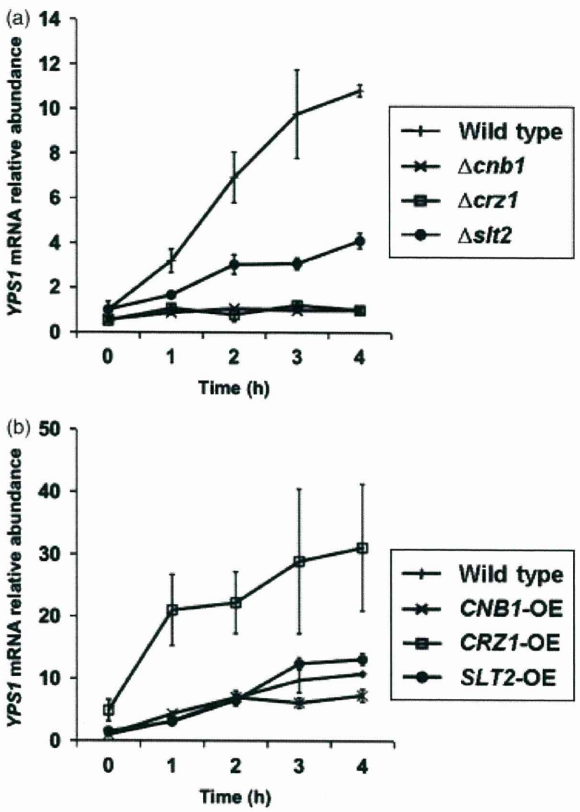

Fig. 2. Time-course analysis of *YPS1* expression in mutant (a) and overexpressing (b) strains of *CNB1*, *CRZ1*, and *SLT2* at an elevated growth temperature. Logarithmic-phase cells grown at 30 °C were adjusted to 1×10^7 cells mL^{-1} and then further incubated at 41 °C. Total RNA was extracted at the indicated time points, and *YPS1* mRNA was measured in triplicate by quantitative real-time RT-PCR. Results represent the average of two independent experiments. Error bars are SDs. *Candida glabrata* strains: wild type, strain TG11; Δ*cnb1*, strain TG162; Δ*crz1*, strain TG172; Δ*slt2*, strain TG152; *CNB1*-OE, a *CNB1*-overexpressing strain (TG163); *CRZ1*-OE, a *CRZ1*-overexpressing strain (TG173); and *SLT2*-OE, an *SLT2*-overexpressing strain (TG153).