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Long-term phlebotomy with low-iron diet therapy lowers risk of development of hepatocellular carcinoma from chronic hepatitis C

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Background. We have previously demonstrated that in patients with chronic hepatitis C (CHC), iron depletion improves serum alanine aminotransferase (ALT) levels as well as hepatic oxidative DNA damage. However, it has not been determined whether continuation of iron depletion therapy for CHC favorably influences its progression to hepatocellular carcinoma (HCC). ***Methods.*** We conducted a cohort study on biopsy-proven CHC patients with moderate or severe liver fibrosis who failed to respond to previous interferon (IFN) therapy or had conditions for which IFN is contradicted. Patients were divided into two groups: subjects in group A (n = 35) underwent weekly phlebotomy (200 g) until they reached a state of mild iron deficiency, followed by monthly maintenance phlebotomy for 44–144 months (median, 107 months), and they were advised to consume a low-iron diet (5–7 mg iron/day); group B (n = 40) comprised CHC patients who declined to receive iron depletion therapy. ***Results.*** In group A, during the maintenance phase, serum ALT levels decreased to less than 60 IU/l in all patients and normalized (<40 IU/l) in 24 patients (69%), whereas in group B no spontaneous decrease in serum ALT occurred. Hepatocarcinogenesis rates in groups A and B were 5.7% and 17.5% at the end of the fifth year, and 8.6% and 39% in the tenth year, respectively. Multivariate analysis revealed that iron depletion therapy significantly lowered the risk of HCC (odds ratio, 0.57) compared with that of untreated patients (P = 0.0337). ***Conclusions.*** Long-term iron depletion for CHC patients is a promising modality for lowering the risk of progression to HCC.

Key words: chronic hepatitis C, phlebotomy, low-iron diet therapy, iron depletion therapy

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Introduction

Hepatitis C virus (HCV) infection affects more than 170 million people worldwide and is a major cause of chronic hepatitis C (CHC), cirrhosis, and hepatocellular carcinoma (HCC) in most developed countries.[1–3] Follow-up studies on the natural history of CHC over a mean period of 4–11 years have showed that cirrhosis develops in 8%–46% of cases and HCC in 11%–19%.[2–4] At present, a combination of pegylated interferon (Peg-IFN) and ribavirin has been established as standard antiviral therapy for CHC.[5] With this therapy, more than 80% of patients with HCV genotype 2 or 3 achieve a sustained virological response (SVR), but among patients with genotype 1, the most common type in many developed countries, including the United States, countries of northern Europe, and Japan, only 50% achieve SVR.[3,6] In addition, IFN therapy is contraindicated in many patients because of complications such as uncontrolled depressive illness, autoimmune hepatitis, and poorly controlled diabetes.[7] Accordingly, other approaches need to be pursued for managing patients who fail to respond to IFN therapy or for whom IFN therapy is not feasible. For such patients with CHC, therapeutic intervention to prevent or delay progression to lethal disease states, such as liver cirrhosis and HCC, are urgently needed.

Recent studies have shown that excess hepatic iron accumulation in CHC patients contributes to liver injury.[8–10] Free iron in the liver is believed to facilitate the formation of reactive oxygen species (ROS), including hydroxyl radicals (·OH), which cause oxidative damage of numerous cellular components such as lipids, proteins, and nucleic acids, and also upregulate collagen synthesis.[11] Further, the ·OH radical is known to generate promutagenic bases such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), which has been implicated in spontaneous DNA mutagenesis and carcinogenesis.[12,13] Although the mechanism of hepatocarcinogene-

sis after HCV infection remains unclear, long-term follow-up studies indicate that most patients with progressive liver diseases who develop cirrhosis or HCC have persistently elevated or fluctuating serum alanine aminotransferase (ALT) levels, suggesting that they have a background of chronic active liver inflammation and regeneration.[14] Hepatocellular damage from HCV infection may be initiated by various immunological reactions occurring on the cell surface, including interactions between Fas on hepatocytes and Fas ligand on cytotoxic T cells and between tumor necrosis factor (TNF) receptors on hepatocytes and TNF released from macrophages and other sources, which subsequently lead to apoptosis caused by ·OH radicals generated in the presence of ferrous iron via a Fenton-type reaction.[15] Thus, iron depletion of hepatocytes can theoretically prevent the generation of toxic ROS and may interfere with apoptotic signaling as well as oxidative DNA damage. In fact, we previously demonstrated that HCC can be completely prevented by a low-iron diet in LEC rats, which accumulate abnormally high levels of copper and iron in the liver and frequently develop HCC, suggesting that iron depletion is effective in decreasing oxidative DNA damage.[16] Further, we also demonstrated in a 6-year follow-up study of CHC patients that iron depletion therapy, consisting of intermittent phlebotomies and a low-iron diet, significantly reduced serum ALT levels, the histological hepatic fibrosis grade, and hepatic 8-OHdG levels.[10] However, it has not yet been determined whether continuation of iron depletion therapy for CHC can favorably influence the progression of disease to HCC.

Therefore, we conducted a cohort study of CHC patients with moderate to severe hepatic fibrosis (a population known to be at high risk for HCC) and examined whether iron depletion therapy could prevent progression to HCC. As a result, we here demonstrate that long-term iron depletion therapy for CHC patients is a promising modality for lowering the risk of progression to HCC.

Methods

Patients

The study enrolled 75 patients with biopsy-proven CHC who routinely visited the liver unit of our department between 1994 and 1996, and who met the following criteria: (1) adult (≥20 years of age) with detectable anti-HCV antibodies and HCV-RNA; (2) previously treated with IFN therapy without achieving SVR or refusal/inability to receive IFN therapy; (3) persistently elevated serum ALT levels (≥60 IU/l for >6 months) at least 1 year after IFN treatment; (4) histopathological evidence of chronic hepatitis with moderate to severe grade liver fibrosis (F2 or F3) on liver biopsy; (5) negative test result for anti-nuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, and hepatitis B surface antigen; (6) no habitual drug or alcohol use; and (7) no administration of ursodeoxycholic acid or glycyrrhizin. The subjects consisted of 38 men and 37 women aged 34 to 75 years with a median age of 60 years. Patients with cirrhosis or a possible HCC association at the time of diagnosis of CHC were excluded from the study. Among the 75 patients, 35 (46.7%) received iron depletion therapy (iron depletion group) after providing written informed consent. The remaining 40 patients were those who declined to receive iron depletion therapy (control group).

Iron depletion therapy

Therapeutic iron depletion was accomplished by performing intermittent phlebotomies in combination with regulation of dietary iron intake as described previously.[10] In brief, at the initial phase of iron depletion, all patients underwent a weekly phlebotomy of 200 g until a state of mild iron deficiency was achieved (defined by either by a serum ferritin concentration of <10 µg/l or a blood hemoglobin concentration of 11.0 g/dl). The mild iron deficiency state was maintained by additional phlebotomies during the study period: patients were followed-up every 1 to 2 months for the duration of the study, and a phlebotomy (200 g) was performed if the hemoglobin level exceeded 11.0 g/dl. In addition, the iron depletion group subjects were instructed, both orally and in writing, by a registered dietitian (SK) to reduce their intake of iron-rich foods during the intervention. To aid with compliance, each subject was given a comprehensive list of iron-rich foods to avoid, and instructions on how to complete dietary records, which required the listing of all food and drink consumed over a 3-day period once every 3 months throughout the intervention. The subjects were not required to alter their total caloric intake, but they were expected to replace iron-rich foods with appropriate substitutes. All patients allocated to the low-iron diets were instructed to reduce their consumption of beans, shellfish, green vegetables, meat, and seaweed, and to replace them with refined carbohydrates. Dietary energy (1900–2000 kcal/day), nutritional balance and iron intake (5–7 mg/day) during the study period were assessed from the dietary records by using the nutrition analysis software Win Kenkoukun-III (Hokenjyouhou, Chiba, Japan).

Laboratory tests

Complete blood cell counts, iron levels (serum iron, serum ferritin, and transferrin saturation), and bio-

chemical parameters, including serum ALT, were determined by using automated procedures in the clinical laboratories of Sapporo Medical University Hospital at every visit. Serum HCV-RNA levels were determined by reverse transcriptase-polymerase chain reaction using a commercial kit (Amplicor HCV; Roche Diagnostics, Branchburg, NJ, USA), and HCV genotypes were assessed by the serological method with a commercial enzyme-linked immunosorbent assay kit (Kokusai Diagnostic, Kobe, Japan).

Liver biopsy

Liver biopsy obtained within 3 months of enrollment was required of all subjects. Biopsy specimens were formalin-fixed, stained with hematoxylin and eosin or Berlin blue, and reviewed by a single pathologist. Hepatic inflammation and fibrosis were scored by using the Knodell histological activity index.[17] Hepatic iron staining was graded by the method of Barton et al.[18]

Follow-up of patients and diagnosis of HCC

Patients was followed up monthly by monitoring hematological and biochemical test results. Computed tomography or ultrasonography was performed every 3 or 4 months in all patients. Angiography was performed when HCC was highly suspected on the basis of computed tomography or ultrasonography imaging.

Statistical analyses

Nonparametric procedures, including the Mann-Whitney *U* test and the χ-squared test, were employed for the analysis of background characteristics. The HCC incidence rate (hepatocarcinogenesis rate) was calculated, based on the period between the diagnosis of CHC by liver biopsy (control group) or start of iron depletion therapy (iron depletion group) and the appearance of HCC, using the Kaplan-Meier technique. Differences in the hepatocarcinogenesis curves were determined by using the log rank test. Independent factors associated with the incidence rate of HCC were analyzed by a stepwise Cox regression analysis. Although continuous variables without data conversion were used in the subsequent multivariate analyses, several variables were transformed into categorical data consisting of two simple ordinal numbers to obtain a hazard ratio. All factors found to be at least marginally associated with liver carcinogenesis ($P < 0.15$) were tested by the multivariate Cox proportional hazard model. $P < 0.05$ was considered to be significant. All data analysis was performed with the computer program SAS.

Results

Patient demographics

At the time of diagnosis of CHC, there were no significant differences between the iron depletion group ($n = 35$) and the control group ($n = 40$) in sex, age, history of blood transfusion, history of IFN therapy, serum albumin concentration, serum bilirubin concentration, serum ALT level, serum γ-glutamyl transpeptidase (GGTP) level, platelet count, serum α-fetoprotein (AFP) concentration, serum type IV collagen level, HCV serotype, HCV concentration, serum ferritin level, stage of liver fibrosis, or hepatic iron deposition score (Table 1). In the iron depletion group, 65.7% of patients had a serum ferritin value exceeding the normal range, and 60.0% in the control group. There was no significant difference between the two groups ($P = 0.779$). In the iron depletion group, Hb levels, serum iron, serum ferritin, and serum ALT levels at the end point were significantly decreased compared with the baseline levels (Table 2).

Crude rates of hepatocarcinogenesis

During the observation period of 144 months, HCC developed in 17/75 patients (22.7%): 4/35 (11.4%) in the iron depletion group, and 13/40 (32.5%) in the control group. As shown in Fig. 1, hepatocarcinogenesis rates in the iron depletion group and control group were 0% and 5.0% at the end of the third year, 5.7% and 17.5% at the end of the fifth year, and 11.8% and 39.0% at the end of tenth year. Log rank tests showed that the hepatocarcinogenesis rate in the iron depletion group was significantly lower than that in the control group ($P = 0.0182$).

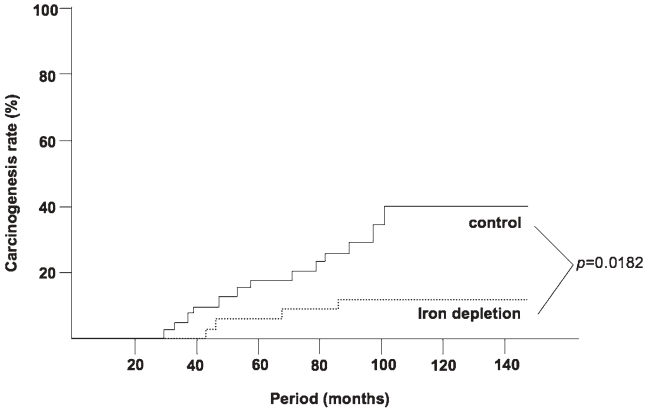

Fig. 1. Crude hepatocarcinogenesis rate in iron depletion and control groups. The carcinogenesis rate was significantly lower in the iron depletion group than in the control group (log rank test)

Table 1. Patient profiles

Factor	Iron depletion	Control	*P* value
Demographics			
Number of patients	35	40	
Sex (M/F)	17/18	21/19	0.912
Age (years)	61 (34–75)	58 (35–75)	0.523
History of blood transfusion	14 (40%)	10 (25%)	0.255
History of interferon therapy	20 (57%)	23 (58%)	0.975
Observation period (months)	114 (44–144)	106 (31–144)	0.411
Laboratory data			
Albumin (g/dl)	4.2 (3.9–5.0)	4.2 (3.5–4.9)	0.992
Bilirubin (mg/dl)	0.7 (0.4–2.1)	0.7 (0.3–1.2)	0.564
ALT (IU/l)	96 (60–421)	87 (60–278)	0.143
GGTP (IU/l)	40 (16–186)	49 (15–224)	0.912
Platelet count ($\times 10^3/\mu l$)	141 (71–197)	142 (84–192)	0.445
AFP (ng/ml)	5.5 (1.5–139)	5.4 (1.5–220)	0.553
Type IV collagen (ng/ml)	150 (83–362)	144 (42–342)	0.465
HCV serotype			
Group 1	30 (86%)	37 (92%)	0.568
Group 2	5 (14%)	3 (8%)	
HCV concentration			
High	32 (91%)	37 (93%)	0.872
Low	5 (9%)	3 (7%)	
Ferritin (ng/ml)	371 (77–1150)	178 (3–1506)	0.971
Histological grade			
Moderate fibrosis	18 (51%)	22 (55%)	0.942
Severe fibrosis	17 (49%)	18 (45%)	
Iron deposition	4 (0–26)	4.5 (0–24)	0.719

ALT, alanine aminotransferase; GGTP, γ-glutamyl transpeptidase; AFP, α-fetoprotein; HCV, hepatitis C virus

Table 2. Changes in iron-related parameters after iron depletion therapy

	Iron depletion group		Control	
Variable	Baseline	End point	Baseline	End point
Hemoglobin (g/dl)	13.9 (11.8–15.2)	10.7 (9.8–11.7)*	13.7 (11.2–15.4)	13.8 (11.1–15.6)
Serum iron (μg/dl)	152 (57–247)	26 (12–40)*	120 (22–321)	142 (36–441)
Serum ferritin(μg/l)	371 (77–1150)	8 (4–21)*	178 (3–1506)	184 (12–1550)
Serum ALT (IU/l)	96 (60–421)	28 (15–65)*	87 (60–278)	84 (58–312)

Data represent medians (range). *$P < 0.01$ vs. baseline

Risk factors affecting hepatocarcinogenesis

Factors associated with hepatocarcinogenesis were analyzed by Cox regression analysis in all 75 CHC patients to determine the effect of iron depletion therapy on disease progression. The univariate analysis showed that the following four factors significantly affected the crude hepatocarcinogenesis rate in all patients: sex ($P = 0.0058$), age ($P = 0.0328$), iron depletion therapy ($P = 0.021$), and platelet count ($P = 0.0131$) (Table 3). Multivariate regression analysis was then performed with these four statistically significant variables. As a result, three factors were significantly and independently associated with hepatocarcinogenesis. The relative risk for hepatocarcinogenesis was greater in male patients than in female patients [odds ratio (OR) = 2.07], in patients ≥60 years old compared with those <60 years old (OR = 1.72), and in patients who did not receive iron depletion compared with in control patients (OR = 1.76) (Table 4).

Serum ALT and serum ferritin levels and hepatocarcinogenesis rates

In the iron depletion group, the average serum ALT level during the maintenance period declined to less than 60 IU/l in all patients and became normal (<40 IU/l) in 24 patients (69%). Figures 2 and 3 show the cumulative hepatocarcinogenesis rates based on

Table 3. Univariate analysis of factors associated with hepatocarcinogenesis in patients with chronic hepatitis C

Factors	Category	Odds ratio (95% CI)	*P* value
Demographics			
Sex (M/F)	Female	1	0.00580
	Male	2.21 (1.26–4.60)	
Age (years)	<60	1	0.0328
	≥60	1.78 (1.06–4.81)	
History of blood transfusion	(+)	1	0.782
	(−)	1.07	
History of interferon therapy	(−)	1	0.735
	(+)	1.08	
Treatment			
Iron depletion	(+)	1	0.0210
	(−)	1.86 (1.11–3.52)	
Laboratory data			
Albumin (g/dl)	≥4.0	1	0.628
	<4.0	1.05	
Bilirubin (mg/dl)	≤1.2	1	0.262
	>1.2	1.51	
ALT (IU/l)	<100	1	0.591
	≥100	1.15	
GGTP (IU/l)	<50	1	0.533
	≥50	1.16	
Ferritin (ng/ml)	<50	1	0.455
	≥50	1.07	
Platelet count	$\geq 10 \times 10^4$	1	0.0131
	$< 10 \times 10^4$	1.73 (1.04–3.06)	
AFP (ng/ml)	<8.5	1	0.220
	≥8.5	1.88	
Type IV collagen (ng/ml)	<150	1	0.826
	≥150	1.21	
HCV serotype	2	1	0.415
	1	1.50	
HCV concentration	Low	1	0.897
	High	1.06	
Histological stage	Moderate fibrosis	1	0.213
	Severe fibrosis	1.35	

CI, confidence interval

Table 4. Multivariate analysis of factors associated with hepatocarcinogenesis in patients with chronic hepatitis C

Factor	Category	Odds ratio (95% CI)	*P* value
Sex (M/F)	Female	1	0.00980
	Male	2.07 (1.18–4.35)	
Age (years)	<60	1	0.0483
	≥60	1.72 (1.00–3.27)	
Iron depletion	(+)	1	0.0337
	(−)	1.76 (1.05–3.33)	
Platelet count	$\geq 10 \times 10^4$	1	0.1493
	$< 10 \times 10^4$	1.73 (0.85–2.56)	

average serum ALT (Fig. 2) and ferritin (Fig. 3) levels, respectively, in patients who underwent iron depletion therapy. The hepatocarcinogenesis rate in patients with serum ALT ≤ 40 IU/l or serum ferritin ≤20 ng/ml was significantly lower than that in patients with serum ALT > 40 IL/l ($P = 0.0377$) or serum ferritin >20 ng/ml ($P = 0.0057$). When a cutoff value for serum ferritin of ≤10 ng/ml was used, the hepatocarcinogenesis rate was also significantly ($P = 0.017$) lower than that in patients with serum ferritin >10 ng/ml. Furthermore, the multivariate

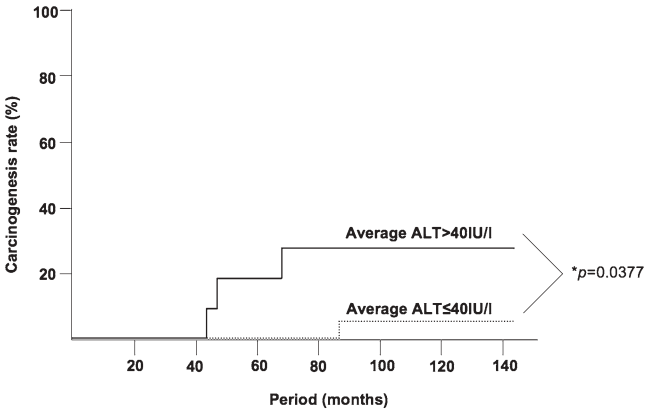

Fig. 2. Hepatocarcinogenesis rate in the iron depletion group. The rate in patients with serum alanine aminotransferase (*ALT*) of ≤40 IU/l was significantly lower than in those with serum ALT > 40 IU/l (log rank test)

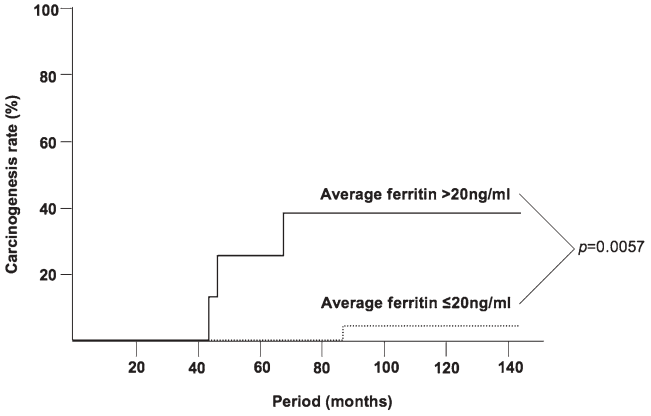

Fig. 3. Hepatocarcinogenesis rate in the iron depletion group. The rate in patients with serum ferritin ≤20 ng/ml was significantly lower than in those with serum ferritin >20 ng/ml (log rank test)

Cox regression analysis indicated that the average serum ferritin level independently affected the hepatocarcinogenesis rate and the relative risk of HCC (OR = 3.48 for patients with average serum ferritin levels of >20 ng/ml vs. those with levels of ≤20 ng/ml).

Discussion

We previously demonstrated in a 6-year cohort trial that CHC patients who received iron depletion therapy showed significant improvements in serum ALT levels, histological hepatic fibrosis score, and hepatic 8-OHdG levels.[10] However, it was unclear at the time whether continuation of iron depletion therapy for CHC could decrease the risk of HCC development. Ideally, to tests such a hypothesis, a prospective randomized trial with an observation period of at least 10 years would be required. However, such a prospective trial would be ethically questionable, because during the study period new effective treatments might emerge for HCV patients who do not respond to IFN therapy, and it would be unethical to withhold such treatment from the study participants, particularly those in the control group. In fact, in the last 10 years, Peg-IFN/ribavirin therapy has become a standard treatment for CHC,[5,6] and clinical trials are currently evaluating other new antiviral agents such as VX-950, a novel inhibitor of HCV NS3.4A serine protease, and siRNA for HCV.[19,20]

Therefore, we conducted a cohort study of two groups of CHC patients over a 12-year observation period. The two groups were comparable because no significant differences were observed between them with respect to parameters such as sex, age, serum ALT levels, history of previous IFN treatment, or liver histological stage (Table 1). The study results clearly indicated that the incidence of HCC development in iron-depleted patients (0.9% per year) was significantly lower than that in control patients (3.9% per year). This reduced incidence of HCC in iron-depleted patients is reasonably low even when compared with previously reported rates of HCC development in CHC patients with moderate to severe fibrosis (2.7%–3.8% per year).[4]

A practical difficulty of the present study was the establishment of an effective protocol to maintain low hepatic iron levels over the study period. Although the mechanism underlying hepatic iron accumulation in patients with CHC remains unclear, excess hepatic iron is considered to be derived from daily food intake,[10] and if dietary iron intake is not restricted, phlebotomy can lead to enhanced iron absorption. Thus, the protocol we adopted for iron depletion therapy was phlebotomy combined with a low-iron diet. This protocol was well tolerated during the study. We advised the patients to reduce their daily iron intake to a maximum of 7 mg, and the actual daily iron intake levels were found to be 5.6 mg/day (range, 5.0–6.8 mg/day), which is approximately half the average dietary iron intake in Japan (11–12 mg per day according to a national nutrition investigation by the Ministry of Welfare of Japan conducted in 1996). As a result, mean serum ferritin levels were kept low and we were able to greatly reduce the mean number of phlebotomies during the maintenance phase. However, in some patients, dietary guidance was not successful and their serum ferritin levels were >20 ng/ml. In such patients, serum ALT levels also fluctuated upward. Hence, multivariate analysis demonstrated that both serum ferritin levels ≤ 20 ng/ml and serum ALT levels ≤ 40 IU/l were independent risk factors for HCC development in the iron depletion group. Accordingly, strict adherence to a low-iron diet is essential for a successful outcome with this treatment modality.

Another practical concern with this modality is its inapplicability to anemic patients (e.g., those with liver cirrhosis) with normal or high serum ferritin levels. In the future, this limitation could conceivably be overcome by using erythropoietin in combination with iron depletion therapy or by using an iron-chelating agent. Nonetheless, iron depletion therapy appears to be a safe and promising means of preventing the progression of CHC to HCC, and is suitable for use until such time as more powerful and less toxic antiviral agents are developed.

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