Title page

Title: Persistent elevated C-reactive protein after treatment is an independent marker of a poor prognosis in patients with hepatocellular carcinoma

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Abstract

Purpose: The pretreatment C-reactive protein (CRP) level is reported to be a prognostic indicator in patients with hepatocellular carcinoma (HCC).

Methods: We investigated the prognostic implications of the changes in the CRP level after initial treatment in patients with HCC. We prospectively evaluated a cohort of 150 patients with newly diagnosed HCC. The patients were divided into three groups: group 1 (n = 120) with pre- and post-treatment CRP< 1.0 mg/dl, group 2 (n = 5) with pre-treatment CRP $\geq$ 1.0 mg/dl and post-treatment CRP < 1.0 mg/dl, and group 3 (n = 25) with pre- and post-treatment CRP $\geq$ 1.0 mg/dl.

Results: The 1-year and 3-year overall survival rates were 92.3 % and 82.9 % for group 1, 80.0 % and 53.3 % for group 2, and 58.8 % and 4.2 % for group 3. The overall survival rate for group 3 was significantly lower than that for group 1 ($P < 0.0001$), or group 2 ($P = 0.003$). No significant difference was found between groups 1 and 2 ($P = 0.627$). A multivariate analysis showed that albumin ($P = 0.049$), the CRP group ($P < 0.0001$), and the Cancer of the Liver Italian Program (CLIP) score ($P < 0.0001$) were independently associated with the overall survival.

Conclusions: A persistently elevated C-reactive protein level after initial treatment is an

independent marker of a poor prognosis, and normalization of the CRP level after initial treatment is associated with a better outcome in patients with HCC.

Keywords

C-reactive protein, Hepatocellular carcinoma, Inflammation, Prognostic marker

Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide, and the third most common cause of cancer-related mortality [1]. It has been estimated that up to 60% to 70% of patients with HCC present with intermediate to advanced stage disease at diagnosis. The survival of patients with unresectable HCC is very poor, with a 3-year survival rate of 8%-10% [2,3]. Quantitative measurement of serum alpha-fetoprotein (AFP) has been conventionally performed as a simple and clinically useful method for routine surveillance, noninvasive diagnosis, monitoring the response to therapy, and evaluating prognosis [4]. However, the diagnostic and prognostic significance of AFP in HCC still remains controversial.

In contrast, increasing evidence supports the involvement of systemic inflammation in diverse pathological conditions, such as cancer, cardiovascular disease and metabolic disease [5]. A systemic inflammatory response can be assessed by measuring the concentration of acute phase proteins, such as C-reactive protein (CRP), fibrinogen, ferritin, albumin and transferring, or peripheral blood leukocyte components, including neutrophils and lymphocytes. Among them, the CRP level has been the most intensively assessed in prognostic studies [6]. Previous studies regarding esophageal carcinoma

[7], gastric carcinoma [8], and colorectal carcinoma [9,10], demonstrated that an elevated serum CRP level was significantly correlated with unfavorable tumor factors, such as distant metastasis, large tumor size, lymph node metastasis, vascular invasion, and tumor recurrence, leading to a poor prognosis.

Consistent with the reports of other cancers, an elevated pretreatment serum CRP level is an independent and significant prognostic indicator in patients with HCC after curative resection [11], and in patients with HCC in various stages of disease and with different liver functional status [12]. However, to our knowledge, no study to date has assessed the association between the changes in CRP following treatment and the oncological outcome in patients with HCC.

Therefore, we set up a prospective study to elucidate the prognostic implications of the changes in the CRP level after treatment in patients with HCC at various stages of disease and with different liver functional status.

Materials and Methods

Patients

Two hundred and eight patients with newly diagnosed HCC who were treated at the Department of Gastroenterology and Hepatology, The Jikei University Daisan Hospital,

between January 2005 and October 2011 were prospectively enrolled. All medical records were reviewed retrospectively. Twenty-three patients were lost to follow-up. Thirty-five patients whose entire set of laboratory data were not available were excluded from this study. Patients who showed clinical evidence of infection or other inflammatory conditions were excluded. In total, 150 patients with HCC were finally included and evaluated. All of these patients were included in a previous study [12].

The diagnosis of HCC was confirmed pathologically or based on images obtained by 4-phase multidetector computed tomography (CT), or dynamic contrast-enhanced magnetic resonance imaging (MRI). The diagnosis should be based on the typical hallmarks of HCC (hypervascularity in the arterial phase with washout in the portal venous or delayed phases) [13]. Tumor-related variables, such as the maximal tumor diameter, tumor number, vascular invasion, and extra hepatic metastases were evaluated by these imaging techniques. The clinical stage (TNM classification) was determined according to the Liver Cancer Study Group of Japan [14].

This study complied with the standards of the Declaration of Helsinki and the current ethical guidelines, and was approved by the institutional ethics board of the Jikei University Daisan Hospital. Written informed consent for participation in the study was not obtained from patients, because this study did not report on a clinical trial, and the

data were retrospective in nature and analyzed anonymously.

CRP and other variables

Blood samples were obtained from all patients before treatment. The serum CRP levels were measured by a latex photometric immunoassay from 2005 to 2007, and thereafter by a turbidimetric immunoassay (Mitsubishi Chemical Company, Ltd., Tokyo, Japan). The results of the two methods showed a significant correlation (correlation coefficient [r] = 1.000, y = 0.97x-0.04). We also evaluated the post-treatment serum CRP level obtained at the first follow-up visits for those who survived for more than ten days after treatment for those who died during their hospital stay (median: 30 days after treatment, range: 5-134 days). We divided the patients into three groups according to the combination of their pre- and post-treatment serum CRP levels. Group 1 had pre- and post-treatment CRP levels < 1.0 mg/dl, group 2 had pretreatment CRP levels $\geq$ 1.0 mg/dl and post-treatment CRP levels < 1.0mg/dl, and group 3 had pre- and post-treatment CRP levels $\geq$ 1.0 mg/dl. We defined the cut-off point for CRP as 1.0 mg/dl based on previous reports [11,12].

The overall survival of the three groups was compared using the clinicopathological variables as follows. The host-related variables were: age, sex, viral markers, levels of

aspartate aminotransferase (AST), alanine aminotransferase (ALT), total serum bilirubin, and albumin, the white blood cell count (WBC), neutrophil to lymphocyte ratio (NLR), platelet count (Plt), prothrombin time (PT), and the Child-Pugh grade. The tumor-related variables were the maximum tumor diameter, number of tumors, extra hepatic metastases, α-fetoprotein level (AFP), the Cancer of the Liver Italian Program (CLIP) score [15], and the clinical stage (TNM classification) [14].

Treatment and patient's follow-up

The indications for surgical resection were patients with solitary lesions, Child-Pugh grade A, no main portal vein trunk involvement, or no distant metastasis. Radiofrequency ablation (RFA) or percutaneous ethanol injection (PEI) was performed for patients with lesions < 3 cm in size and < 3 in number. Transcateheter arterial chemoembolization (TACE) or Lipiodol- Transcateheter arterial infusion (TAI) was performed for patients with more than 4 multiple lesions or lesions larger than 3 cm in size. Systemic chemotherapy or targeted therapy, including sorafenib, was administered to patients with distant metastasis and preserved liver function. Only the best supportive care (BSC) was given for patients with Child-Pugh grade C or distant metastasis.

Patients were followed carefully after the initial treatment. The serum AFP level was

measured once every month. Ultrasonography and dynamic CT were performed every three months. Selective hepatic arterial angiography or a percutaneous biopsy was performed in patients with suspected tumor recurrence. The start date of the follow-up was the date of the initial diagnosis of HCC. The end of the follow-up was the time of the last follow-up (October 2011) or death.

Statistical analysis

Continuous variables are presented as the medians and ranges. Categorical variables are presented as numbers and percentages. Comparisons between the groups were performed with the Kruskal-Wallis test for continuous and ordinal variables and with the chi-square test for categorical variables. The overall survival rates were calculated using the Kaplan-Meier method and differences in the survival rates between the groups were compared by the log-rank test. A univariate and multivariate analysis were performed for the prognostic factors using the Cox-proportional hazard model. Variables that proved to be significant in the univariate analysis were tested subsequently with the multivariate Cox-proportional hazard model. The forward selection method was used for the multivariate Cox-proportional analysis. A P-value < 0.05 was considered to be significant. All statistical analyses were performed using the IBM SPSS Statistics

software v.19.0 software program (IBM SPSS Inc., Chicago, IL, USA).

Results

Patients characteristics

The baseline characteristics of the patients are shown in **Table 1**. The median age of the patients was 72 (range 43-91) years. One hundred and six (70.7%) patients were males and 44 (29.3%) patients were females. Eighty-four (56%) patients were positive for antibodies to hepatitis C virus (anti-HCV), and 20 (13.3%) patients were positive for hepatitis B surface antigen (HBs Ag). One hundred and seven patients (71.3%) had preserved liver function (Child-Pugh A Grade), and 78 patients (52%) were classified as having Stage I or II disease. Surgical resection was performed in nine (6%) patients, TACE or RFA were administered to 134 (89.3%) patients. The remaining seven (4.7%) patients received BSC.

The median pretreatment serum CRP level was 2 mg/l, ranging from 1 to 188 mg/l. One-hundred and twenty (80%) patients were allocated to group 1 (pre- and post-treatment CRP < 1.0 mg/dl), five (3.3%) patients were allocated to group 2 (pre-treatment CRP $\geq$ 1.0mg/dl and post-treatment CRP < 1.0 mg/dl), and 25 (16%) patients were allocated to group 3 (pre- and post-treatment CRP $\geq$ 1.0mg/dl).

There was a significant correlation between the pretreatment serum CRP level and the NLR ($r = 0.399$, $P < 0.0001$) (**Fig. 1**). A box - plot indicating the distribution of the pretreatment serum CRP level and the clinical stage is presented in **Fig. 2**.

The clinicopathological characteristics of the group 2 and group 3 patients are shown in **Table 2**. There were no significant differences between these two groups regarding the host-related variables and tumor-related variables.

Survival

The median duration of follow-up was 18 (range 1-80) months. Seventy-seven (51.3 %) patients were alive at the end of the follow-up period, and 73 (48.7 %) patients had died. The 1-year, 3-year and 5-year overall survival rates were 74.1 %, 53.3 %, and 28.4 %, respectively.

The comparison of overall survival according to the groups is shown in **Fig. 3**. The 1-year and 3-year overall survival rates were 92.3 % and 82.9 % for group 1, 80.0 % and 53.3 % for group 2, and 58.8 % and 4.2 % for group 3, respectively. The overall survival rate for group 3 was significantly lower than that for group 1 ($P<0.0001$) and for group 2 ($P=0.003$). However, no significant difference in survival was found between groups 1 and 2 ($P=0.627$).

Prognostic factors

The univariate analysis showed that the AST level (P = 0.001), total serum bilirubin (P < 0.0001), albumin (P < 0.0001), CRP group (P < 0.0001), AFP (P < 0.0001), Child-Pugh grade, CLIP score (P < 0.0001), clinical stage (P < 0.0001), maximal tumor diameter (P < 0.0001), multiple nodules (P < 0.0001), vascular invasion (P < 0.0001), extrahepatic metastasis (P = 0.001) were associated with the overall survival (**Table 3**).

A multivariate analysis of these significant variables showed that the albumin level (HR 1.668, 95%CI 1.001-2.778, P = 0.049), CRP group (HR 1.976, 95%CI 1.439-2.714, P < 0.0001), and CLIP score (HR 2.191, 95%CI1.739-2.76, P < 0.0001) were independently associated with the overall survival (**Table 3**).

Discussion

In this study, we have demonstrated that the persistent elevation of the CRP level after initial treatment is an independent marker of a poor prognosis in patients with HCC, and that the normalization of the CRP level after initial treatment is associated with an improved outcome in patients with HCC.

Several possible mechanisms have been proposed for the elevation of the CRP level in

cancer patients. First, cancer growth could induce a tissue inflammatory response and thus increase the CRP level because inflammation has been shown to play an important role in the pathogenesis and progression of many types of cancer [16].Second, the CRP level might reflect an inflammatory response activated as a secondary process due to tumor necrosis or local tissue damage. Third, cancer cells themselves could increase the production of inflammatory cytokines, such as IL-6 and IL-8, which in turn would induce the production of CRP [5].

The mechanism by which a systemic inflammatory response reflected by an elevation of the serum CRP or IL-6 levels might influence survival in patients with cancer is not clear. However, it has been known that the release of pro-inflammatory cytokines and growth factors, some of which cause metabolic disturbance and a decrease in lean tissue, plays some role in this systemic reaction. The presence of inflammatory factors may also promote tumor growth, which, in turn, may further stimulate the systemic inflammatory response [10,17].

Moreover, recent reports demonstrated that elevated serum CRP levels were also associated with several other prognostic factors, such as the presence of distant metastasis, tumor size, lymph node metastasis, vascular invasion, and tumor recurrence [7-10].

In fact, there is increasing evidence that the presence of a systemic inflammatory response, as evidenced by an elevated CRP level or elevated neutrophil to lymphocyte ratio (NLR), is associated with poor survival in patients with various malignancies, including HCC [11,12,18,19].

Recently, Chua et. al. demonstrated that a systemic inflammatory response, as evidenced by an elevated NLR, can be reversed by treatment, and that normalization of the NLR shortly after the commencement of chemotherapy is an early predictor of a significant improvement in survival in patients with advanced colorectal cancer [20].

In the setting of HCC, Chen et. al. have demonstrated that a persistently elevated NLR after radiofrequency ablation is associated with a worse outcome in patients with HCC [21]. Pinato and Sharma have also shown that a persistently elevated NLR after transarterial chemoembolization is associated with a worse outcome in patients with HCC [22]. However, the cut-off points used for the NLR in these studies were different, and no optimal cut-off point has been determined. Moreover, these results conflict with a previous report by Huang indicating that HCC patients with a persistently elevated NLR after transarterial chemoembolization had a better outcome than those with a decreased NLR [23].

In a previous study, we demonstrated that an elevated pre-treatment CRP level is associated with tumor progression and reduced liver function, and can be considered an independent marker of a poor prognosis in patients with HCC, irrespective of the tumor stage and liver function [12]. In the current study, we demonstrated that a persistently elevated CRP level after initial treatment is independently associated with a worse outcome in patients with HCC. These results are consistent with previous reports indicating that a persistent deranged inflammatory response is an independent poor prognostic indicator in patients with HCC [21,22]. We also have shown that patients with normalization of the CRP level after treatment have a survival advantage over those with a persistently elevated CRP level, suggesting that CRP can serve as a surrogate marker for the treatment outcome.

In the current study, the multivariate analysis revealed that the CRP group can be considered an independent factor predicting a poor prognosis. In contrast, the AFP level was not found to be independent prognostic factor. Serological tests, including AFP, des-gamma-carboxy prothrombin (DCP), the ratio of glycosylated AFP (L3 fraction) to total AFP, alpha-fucosidase, and glypican 3 have been investigated for the surveillance, diagnosis, prognostic evaluation, and monitoring the response to treatment in patients with HCC [13]. Among these factors, AFP is the most widely examined biomarker.

However, recent studies have shown that AFP determination lacks adequate sensitivity and specificity for effective surveillance, diagnosis, and dose not have prognostic significance [24-26]. In fact, the current guidelines for the management of HCC state that none of the serological tests can be recommended to survey patients at risk of developing HCC, and that surveillance has to be based on ultrasound examination [13,27].

Inflammation-based prognostic markers, such as CRP, have the advantage of being simple, and do not require additional imaging techniques or histological examinations. They are inexpensive, readily available, and can be measured repeatedly. In this regard, CRP can be clinically applicable as a marker for prognostication and for monitoring the response to treatment in patients with HCC.

The current study has some limitations. First, the duration between the initial treatment and the time when data were obtained after the initial treatment varied between patients, leading to heterogeneity in the measurement of the CRP level. However, the optimal timing for the measurement of the CRP level after treatment has not been elucidated. Therefore, a large-scale prospective validation study is needed to determine the optimal timing. Second, the therapeutic effects of the second and third lines of treatments for HCC were not evaluated as prognostic factors. Since many

patients received multiple treatment sessions due to HCC recurrence during their follow-up, it was considered to be difficult to evaluate all the therapeutic effects as prognostic factors in this patient population.

Conclusions

The current study has demonstrated that the persistent elevation of the CRP level after initial treatment is an independent marker of a poor prognosis in patients with HCC, and that the normalization of the CRP level after initial treatment is associated with an improved outcome. The CRP level can be clinically applicable as a marker for prognostication and of the response to treatment in patients with HCC.

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Conflict of interest statement

The authors disclose no conflict of interest.

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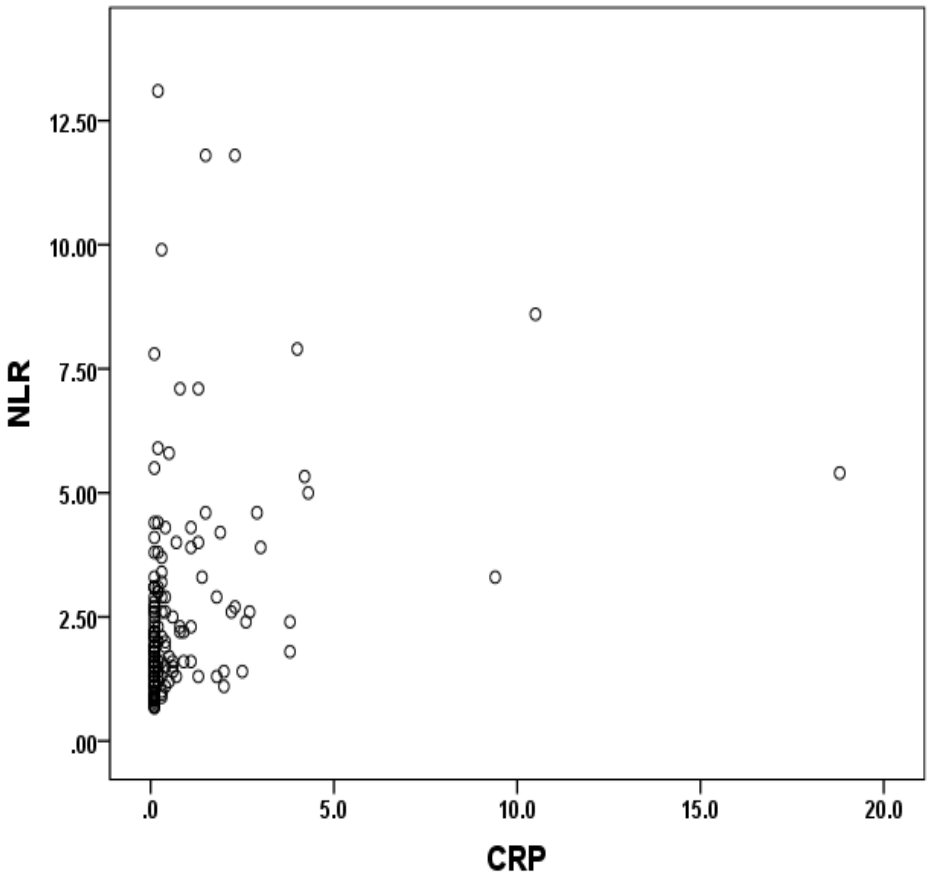
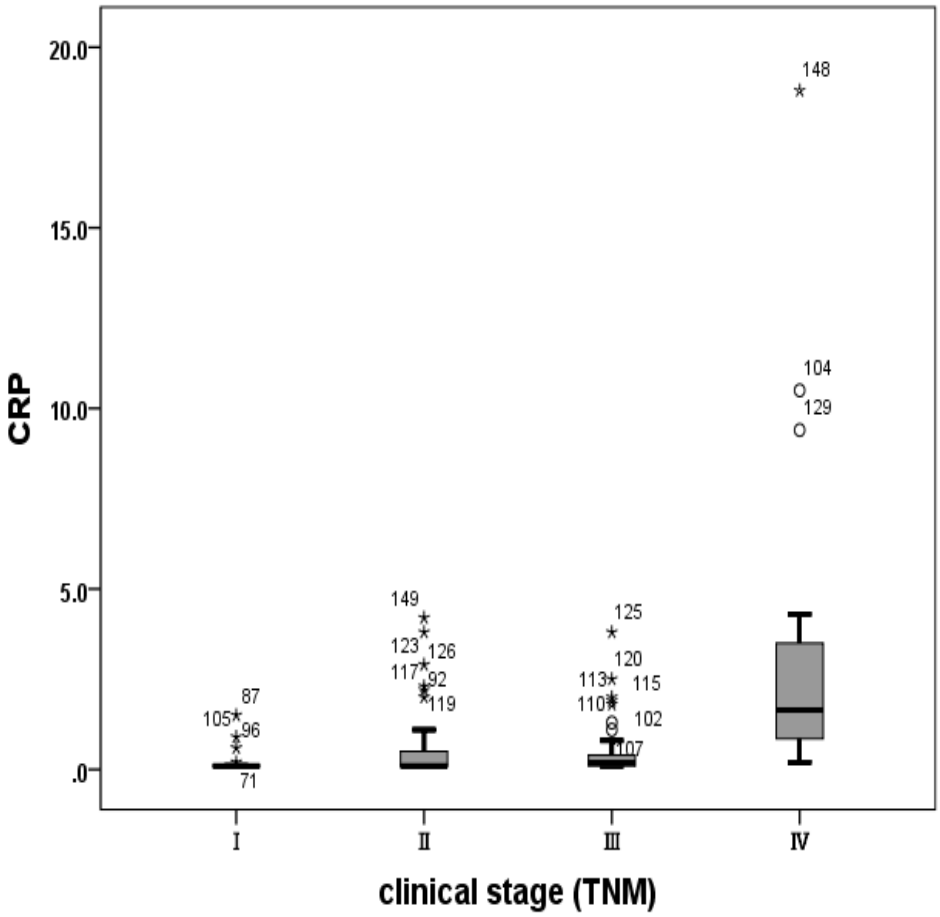


Fig. 1


20.0
15.0
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CRP
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I
II
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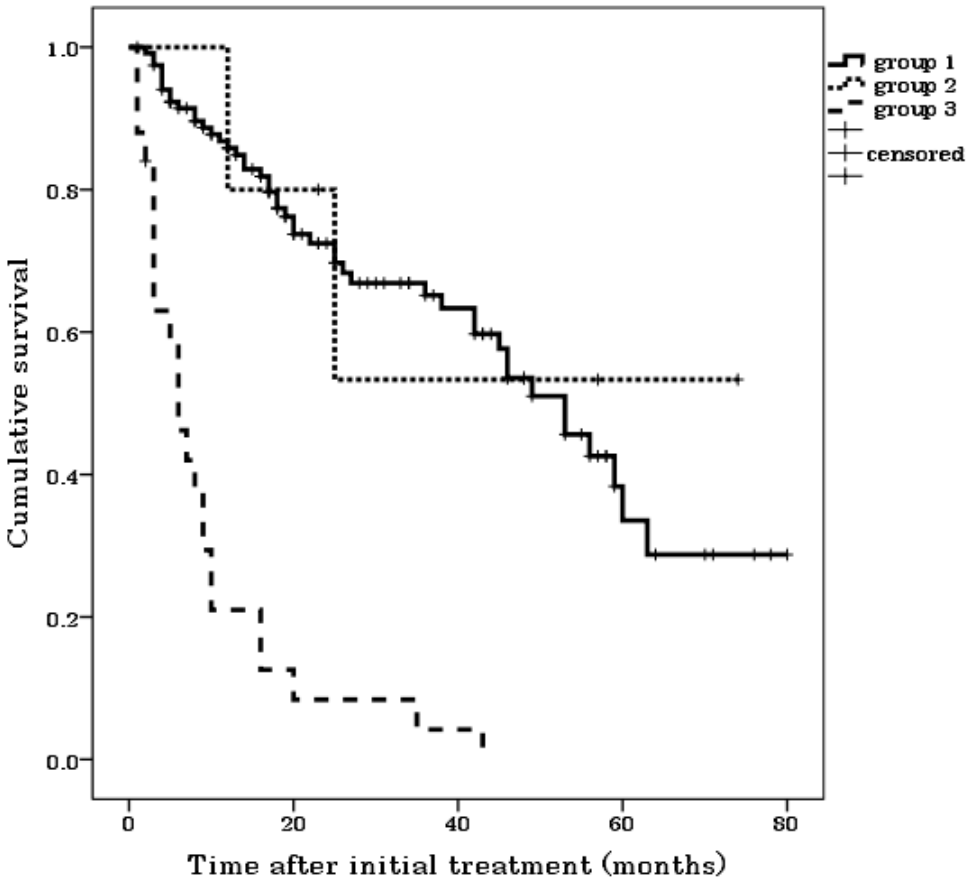
IV
clinical stage (TNM)

Fig. 2


1.0
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0.6
0.4
0.2
0.0
Cumulative survival
0
20
40
60
80
Time after initial treatment (months)
group 1
group 2
group 3
censored

Fig. 3

Figure caption

Fig. 1 A scatter diagram of the pretreatment serum CRP level and NLR.

Fig. 2 A box - plot indicating the distribution of pretreatment serum CRP level and the clinical stage.

Fig. 3 Survival curves according to the CRP group.

Table 1 Clinicopahtological characteristics of the patients

Variables	n=150
Age (years)	72 (43-91)
Sex (male/female)	106/44
HBsAg positive (%)	20 (13.3)
HCVAb positive (%)	84 (56)
AST (IU/l)	55.5 (13-384)
ALT (IU/l)	41.5 (8-202)
Total serum bilirubin (mg/dl)	0.8 (0.3-8.3)
Albumin (g/l)	36 (21-50)
CRP (mg/l)	2 (1-188)
WBC ($\times 10^9$/l)	5.3 (2.5-14.8)
Neutrophil to lymphocyte ratio	2 (0.7-13.1)
Platelet count ($\times 10^4/mm^3$)	13.9 (1.8-44.3)
Prothrombin time (%)	82 (38-100)
α-fetoprotein level (ng/ml)	25.3 (1.7-280600)
Child-Pugh grade (A/B/C)	107/37/6
CLIP score (0/1/2/3/4/5/6)	44/50/28/12/9/5/2
Tumor stage (I,II,III,IV)	22/60/48/20
Maximal tumor diameter (mm)	33 (7-200)
Tumor number (solitary/multiple)	77/73
Vascular invasion (absent/present)	135/15
Extrahepatic metastasis (absent/prsent)	144/6
Initial treatment (resection/TACE (TAI),RFA,PEI/BSC)	9/134/7

Abbreviations: HBsAg = hepatitis B surface antigen; HCVAb = hepatitis C antibody; AST = aspartate aminotransferase; ALT = alanine aminotransferase; CRP = C-reactive protein; WBC = white blood cell count; CLIP = the Cancer of the Liver Italian Program
TACE = transcateheter arterial chemoembolization; TAI = lipiodol- transcateheter arterial infusion; RFA = radiofrequency ablation; PEI = percutaneous ethanol injection; BSC = best supportive care

Table 2 Clinicopathological characteristics of the group 2 and 3 patients

Variables	Group 2	Group 3	P-value
Age (years)	64(56-74)	73(51-82)	0.094
Sex (M/F)	4/1	21/4	0.642
HBsAg or HCVAb (negative/positive)	2/3	13/12	0.465
AST (IU/l)	83(40-258)	99(13-384)	0.707
ALT (IU/l)	81(26-157)	63(8-190)	0.488
Total serum bilirubin (mg/dl)	0.9(0.3-1.3)	1.55(0.3-8.3)	0.157
Albumin (g/l)	33(22-39)	33(22-46)	0.729
CRP (mg/l)	2.7(1.1-10.5)	2.1(1.1-18.8)	0.47
WBC ($\times 10^9$/l)	5.5(3.9-7.3)	6.35(3.5-12.4)	0.248
Platelet count ($\times 10^4/mm^3$)	20.0(8.7-25.3)	18.85(1.8-44.3)	0.356
Prothrombin time (%)	77(63-100)	82.5(45-100)	0.544
α-fetoprotein level (ng/ml)	40.0(7.5-316)	555.5(1.7-280600)	0.419
Child-Pugh grade (A/B/C)	(2/3/0)	(14/7/4)	0.885
CLIP score (0/1/2/3<)	(1/2/0/1/1)	(2/4/4/15)	0.166
Tumor stage (I,II,III,IV)	(0/3/0/2)	(1/4/7/13)	0.386
Maximal tumor diameter (mm)	40.0(25-150)	60(10-150)	0.453
Tumor number (multiple:solitary)	2/3	7/18	0.206
Vascular invasion (absent/present)	3/2	17/8	0.551
Extrahepatic metastasis (absent/prsent)	4/1	22/3	0.538
Initial treatment (resection/TACE (TAI),RFA,PEI/BSC)	1/4	1/16/8	0.186

Abbreviations: HBsAg = hepatitis B surface antigen; HCVAb = hepatitis C antibody; AST = aspartate aminotransferase; ALT = alanine aminotransferase; CRP = C-reactive protein; WBC = white blood cell count; CLIP = the Cancer of the Liver Italian Program; TACE = transcateheter arterial chemoembolization; TAI = lipiodol- transcateheter arterial infusion; RFA = radiofrequency ablation; PEI = percutaneous ethanol injection; BSC = best supportive care

Table 3 Prognostic factors for overall survival in patients with HCC: Results of the univariate and multivariate analyses

	Univariate analysis	Multivariate analysis	
Variables	P-value	Hazard ratio (95% CI)	P-value
Age (<60/≥60)	0.776		
Sex (male/female)	0.352		
HBs Ag or HCV Ab (negative/positive)	0.227		
AST (<2 × normal limit/≥2 × normal limit)	0.001		
ALT (<2 × normal limit/≥2 × normal limit)	0.053		
Total serum bilirubin (<2.0 mg/dl/≥2.0 mg/dl)	<0.0001		
Albumin (≤3.5 g/dl/>3.5 g/dl)	<0.0001	1.668 (1.001-2.778)	0.049
CRP group (1/2/3)	<0.0001	1.976 (1.439-2.714)	<0.0001
WBC	0.112		
Platelet count ($\leq 10\times10^4/mm^3$/$>10\times10^4/mm^3$)	0.12		
Prothrombin time (≤80%/>80%)	0.089		
α-fetoprotein level (<400 ng/ml/≥400 ng/ml)	<0.0001		
Child-Pugh grade (A/B/C)	<0.0001		
CLIP score (0/1/2/3/4/5/6)	<0.0001	2.191 (1.739-2.76)	<0.0001
Tumor stage (I/II/III/IV)	<0.0001		
Maximal tumor diameter (<50 mm/≥50 mm)	<0.0001		
Tumor number (solitary/multiple)	<0.0001		
Vascular invasion (absent/present)	<0.0001		
Extrahepatic metastasis (absent/prsent)	<0.0001		

Abbreviations: HBsAg = hepatitis B surface antigen; HCVAb = hepatitis C antibody; AST = aspartate aminotransferase; ALT = alanine aminotransferase; CRP = C-reactive protein; WBC = white blood cell count; CLIP = the Cancer of the Liver Italian Program