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14) 2014年10月20日～21日　The 1st International Symposium on Mucosal Immunity and Vaccine Development 2014（東京大学）「Nucleic acids as 'built-in' or 'inducible' adjuvant during vaccination」

15) 2014年11月6～7日　The 2014 Fall Conference of The Korean Association of Immunologists「New mechanism of action and potential biomarkers for vaccine adjuvant」

16) 2014年11月10日～12日　第62回日本ウイルス学会学術集会シンポジウム「次世代のワクチン開発～Next generation vaccine development」「New mechanism of action and potential biomarkers for vaccine adjuvant」

17) 2014年12月4日～5日　第12回日本糖鎖科学コンソーシアム（JCGG）シンポジウム「新規アジュバント開発に向けて」

18) 2014年12月4日～5日　第27回日本バイオセラピィ学会学術集会「がん免疫療法に資する核酸医薬を基盤としたアジュバントの開発」

19) 2015年2月3日　琉球大学　講義「アジュバント開発研究の新展開：安全でよく効くワクチンを目指して」

20) 2015年3月25日～28日　日本薬学会第135年会「アジュバント開発研究の最前線：データベースを駆使した安全性、有効性のバイオマーカー」

G. 知的所有権の出願・取得状況

1. 特許取得

1) 発明の名称: 免疫賦活活性を有するオリゴヌクレオチド含有複合体及びその用途
発明者: 石井　健・小檜山　康司・青枝　大貴、武下　文彦・粕谷　祐司・丹羽　貴子・小泉　誠
出願人: 独立行政法人医薬基盤研究所・第一三共株式会社
出願日: 平成26年9月19日
出願番号: 特願2013-196206, PCT/JP2014/074835

2) 発明の名称: 免疫賦活活性を有する核酸多糖複合体の抗腫瘍薬としての応用
発明者: 石井　健・青枝　大貴・小檜山　康司
出願人: 独立行政法人医薬基盤研究所
出願日: 平成26年12月26日
出願番号: PCT/JP2014/084772

2. 実用新案登録

なし

3. その他

特になし

厚生労働科学研究費補助金
[肝炎等克服実用化研究事業（B型肝炎創薬実用化等研究事業）]
分担研究報告書

B型肝炎ウイルス由来細胞傷害性T細胞エピトープの同定とペプチドワクチンの開発

研究分担者：水腰 英四郎　金沢大学医薬保健研究域医学系　准教授

研究要旨：B型肝炎ウイルス（HBV）のcccDNAは、血液中にウイルス粒子が検出されない症例においても、生涯にわたって肝組織中に存在し、現行の抗ウイルス治療に抵抗性である。cccDNAの制御や感染細胞の排除を目的とする治療法を開発するためには、cccDNA感染細胞において発現している細胞傷害性T細胞(CTL)エピトープを明らかにし、より強力な免疫応答を誘導できる治療方法を開発することが必要である。本研究では、こうしたCTLエピトープの同定と同エピトープのアミノ酸配列に基づいたペプチドワクチンの開発を行い、HBV感染細胞の排除を目的とした、より強力な免疫治療法の開発を行う。本年度はペプチドワクチンの候補となるHBV由来CTLエピトープの同定と、より強力なペプチドワクチンを作製するための調整法の検討を行った。

A. 研究目的

HBV cccDNA 感染肝細胞に発現している細胞傷害性 T 細胞エピトープを同定することにより、cccDNA 感染細胞の排除を目的とする免疫治療法の開発を行う。

B. 研究方法

HBV genotype C の large S 領域、pre-core/core 領域、HBx 領域、polymerase 領域のアミノ酸配列を基に、コンピュータソフト(BIMAS)を用いて、HLA-A24 拘束性 CTL エピトープの予測を行う。次に HLA-A24 分子への結合予測スコアが 5.0 以上のエピトープをもつペプチドを作製し、各種免疫学的アッセイ法（ELISPOT アッセイ、CTL アッセイ等）にてヒト末梢血リンパ球での免疫反応を検討する。こうしたエピトープスクリーニングにおいて、免疫治療に有用と考えられるエピトープを選択し、HLA-A24 トランスジェニックマウスを用いて、ペプチドワクチンとしての有用性を検証する。本研究の倫理面への配慮として、臨床研究・疫学研究・ヒトゲノム・遺伝子解析研究に関する倫理指針を遵守する。本研究に関しては、研究施設内の倫理委員会として、１）医学倫理審査委員会と２）ヒトゲノム・遺伝子解析研究倫理審査委員会の２つの承認を得ている。

C. 研究結果

コンピュータにて予測された CTL エピトープのうち、large S 領域から 31 種類、pre-core/core 領域から 14 種類、HBx 領域から 4 種類、polymerase 領域から 44 種類

のエピトープを、結合予測スコアが高い順に選択し、免疫学的解析に用いるためのペプチドを作製した。また、これらのペプチドとヒトリンパ球を用いて、ペプチドに反応しインターフェロンガンマを産生するCTLを検出するためのELISPOTアッセイシステムを構築し、免疫反応を測定した。

これまでに50例のHBV感染患者（HBV既感染者3例、未治療慢性肝炎患者22例、核酸アナログ製剤による治療中の慢性肝炎患者17例、急性肝炎患者2例、肝機能正常キャリアー6例）において末梢血リンパ球の免疫応答の解析が終了しており、93種類のHBV由来ペプチドのうち49種類において、少なくとも1人以上の患者において陽性反応を認めた。また3例以上において陽性反応を認めたペプチドは6種類であり、うち2種類ではペプチドの刺激によりCTLの誘導が可能であった。今回同定されたHBV由来CTLエピトープに対する免疫反応と臨床データを詳細に解析すると、免疫応答を認めた患者では血清ALT値が高く、HBVコア関連抗原量が低いといった、cccDNA感染細胞の除去に関与している可能性のあるエピトープと推定されるものが含まれていた。HBV由来エピトープに特異的なCTLの免疫応答は、核酸アナログによるB型肝炎の治療後において増強していた。

HLA-A24トランスジェニックマウスを用いた検討では、上記エピトープ由来のペプチドワクチンによる免疫誘導効果と安全性が確認された。また、ペプチドワクチンを作製する際のアジュバントとして、モンタナイドISA-51やCpGODNの有用性が明らかになり、両者の併用がワクチンによる免疫誘導効果をより強く誘導できることを証明した。

D. 考察

今回同定した新規HBV由来ペプチドとアジュバントとの組み合わせにより、HBV排除を目的としたペプチドワクチン開発の可能性が示された。今後は、さらに各エピトープに対する免疫反応と細胞内HBVcccDNAとの関連を培養細胞やヒト肝組織で検証するとともに、ペプチドワクチンのヒトでの安全性を検証するための臨床試験が必要と考えられた。

E. 結論

HBVcccDNA感染細胞に対する免疫治療法の開発に必要なペプチドの同定と、動物モデルにおけるペプチドワクチンの免疫誘導効果と安全性を確認した。

F. 研究発表

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2. 学会発表

なし

G. 知的所有権の出願・取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

特になし

III. 研究成果の刊行に関する一覧表

別紙５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
T Terashima,（金子、水腰）	Blood neutrophil to lymphocyte ratio as a predictor in patients with advanced hepatocellular carcinoma treated with hepatic arterial infusion chemotherapy.	Hepatol Res	-	-	(in press)
K Yamada,（金子、水腰）	Characteristics of hepatic fatty acid compositions in patients with nonalcoholic steatohepatitis.	Liver Int	-	-	(in press)
T Yamashita,（金子、中本、水腰）	Gd-EOB-DTPA-enhanced magnetic resonance imaging and alpha-fetoprotein predict prognosis of early-stage hepatocellular carcinoma.	Hepatology	60(5)	1674-1685	2014
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M Kitahara,（金子、中本、水腰）	Efficient generation of highly immunocompetent dendritic cells from peripheral blood of patients with hepatitis C virus-related hepatocellular carcinoma.	Int Immunopharmacol	21(2)	346-353	2014
N Oishi,（金子）	Molecular biology of liver cancer stem cells.	Liver Cancer	3(2)	71-84	2014

F Lan, （金子）	LECT2 functions as a hepatokine that links obesity to skeletal muscle insulin resistance.	Diabetes	63(5)	1649-1664	2014
Y Takeshita, （金子、水腰）	The effects of ezetimibe on non-alcoholic fatty liver disease and glucose metabolism: a randomised controlled trial.	Diabetologia	57(5)	878-890	2014
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Ⅳ.　研究成果の刊行物・別刷

Hepatology Research

Hepatology Research 2014 doi: 10.1111/hepr.12436

Original Article

Blood neutrophil to lymphocyte ratio as a predictor in patients with advanced hepatocellular carcinoma treated with hepatic arterial infusion chemotherapy

Takeshi Terashima, Tatsuya Yamashita, Noriho Iida, Taro Yamashita, Hidetoshi Nakagawa, Kuniaki Arai, Kazuya Kitamura, Takashi Kagaya, Yoshio Sakai, Eishiro Mizukoshi, Masao Honda and Shuichi Kaneko

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Aim: Inflammation plays a critical role in cancer. The aim of the present study was to investigate the impact of neutrophil to lymphocyte ratio (NLR) on patients with advanced hepatocellular carcinoma (HCC) treated with hepatic arterial infusion chemotherapy (HAIC).

Methods: We retrospectively evaluated 266 patients with advanced HCC treated with HAIC between March 2003 and December 2012. NLR was calculated from the differential leukocyte count by dividing the absolute neutrophil count by the absolute lymphocyte count.

Results: The cut-off level of NLR was set as the median value of 2.87 among all patients in this study. The objective response rate in the patients with low NLR was 37.6%, which was significantly better than that of the patients with high NLR (21.1%; $P < 0.01$). Multivariate analysis revealed that low NLR remained associated with the response to HAIC ($P = 0.024$). Median progression-free survival and median overall survival in patients with high NLR were 3.2 and 8.0 months, respectively, which were significantly shorter than that of the patients with low NLR (5.6 and 20.7 months; $P < 0.01$ and $P < 0.01$, respectively). High NLR was an independent unfavorable prognostic factor in multivariate analysis. The patient outcome was stratified more clearly by NLR calculated after HAIC added to calculations before HAIC. Serum platelet-derived growth factor-BB level was positively correlated with NLR.

Conclusion: Results suggest that NLR is a useful predictor in patients with advanced HCC treated with HAIC. These findings may be useful in determining treatment strategies or in designing clinical chemotherapy trials in future.

Key words: hepatic arterial infusion chemotherapy, hepatocellular carcinoma, neutrophil lymphocyte ratio, predictive factor, prognostic factor

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the third leading cause of cancer death and remains a worldwide health concern because the incidence of HCC continues to increase globally.[1] A variety of new techniques of imaging modalities have enabled the detection of HCC at early stages, and advances of various therapeutic procedures have improved the curability of patients with HCC.[2] Despite those recent advances in diagnostic and therapeutic technologies, the prognosis of patients with HCC remains poor due to impaired liver function and frequent recurrence of HCC.[3]

Although sorafenib has been established as the standard of care for advanced HCC,[4] its efficacy and tolerability are limited.[5] As an alternative therapy to sorafenib, hepatic arterial infusion chemotherapy (HAIC) has been conducted in Asia, including Japan, and it has been reported as a promising treatment procedure.[6,7] However, application of HAIC and its predictive and prognostic markers have not been fully established.

Inflammation plays a critical role in the development and progression of various cancers.[8] Inflammation caused by extrinsic factors including a variety of infectious agents and environmental toxins, as well as intrinsic factors including active oncogenes, reactive

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oxygen species and necrosis existing in the cancer tissues, promote various processes of cancer initiation and progression, such as mutation, proliferation, immortalization, invasiveness, angiogenesis, epithelial–mesenchymal transition and immunosuppression.[9] Additionally, the release of inflammation-related substances is closely related to symptoms such as loss of bodyweight, fatigue and appetite loss among cancer patients. Therefore, inflammation-induced cancer progression and cachectic patient status affect quality of life and patient outcomes.[10] The inflammation-related markers such as absolute white blood cell count, C-reactive protein (CRP), neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio and cytokines have been suggested to be associated with outcomes of patients with various malignancies[11] including at an early or intermediate disease stage of HCC.[12–16] However, whether these markers can serve as biomarkers of treatment efficacies and patient outcome in more advanced stages of HCC remains unclear.

The objectives of the present study were to investigate the correlation between NLR and patient characteristics in advanced HCC patients. We also analyzed the impact of NLR on the treatment efficacies as well as the outcome of patients with advanced HCC treated with HAIC. Moreover, to assess inflammatory molecules associated with NLR, serum level of cytokines and growth factors were measured. This approach provides useful information in determining treatment strategies for patients with advanced HCC.

METHODS

Patients

THE SUBJECTS IN this study were patients treated with HAIC at the Kanazawa University Hospital between March 2003 and December 2012 for advanced HCC with vascular invasion and/or intrahepatic multiple lesions considered unsuitable for surgical resection, locoregional therapy and transarterial chemoembolization. All patients underwent dynamic computed tomography (CT) or dynamic magnetic resonance imaging (MRI) to diagnose HCC and assess the extent of cancer. Additionally, HCC was diagnosed according to the guidelines of the American Association for the Study of Liver Disease.[17] Patients with extrahepatic lesions were also considered eligible for HAIC if their extrahepatic lesions were mild; intrahepatic lesions were considered to be prognostic factors. Other inclusion criteria were Eastern Cooperative Oncology Group performance status (ECOG PS) of 2 or less, appropriate major organ functions, including bone marrow, kidney, cardiac functions and hepatic function (Child–Pugh A or B), and no clinical symptoms or signs of sepsis.

HAIC

The technique for implantation of the reservoir system has been thoroughly described elsewhere.[18] Catheters were induced through the right femoral artery and angiography from the celiac artery was first performed to localize the HCC and evaluate the intrahepatic and extrahepatic vascularization. Then, we inserted a catheter with a side opening into the gastroduodenal artery, positioning the side opening in the common hepatic artery by an image-guided procedure. The gastroduodenal artery, right gastric artery and other arteries that were suspected to nourish the gastroduodenal region were embolized as much as possible to prevent the gastrointestinal mucositis. The other end of the catheter was connected to the injection port subcutaneously implanted in the right lower abdomen. Finally, we confirmed blood flow redistribution.

Hepatic arterial infusion chemotherapy was conducted approximately 5 days after the reservoir was implanted. The treatment protocol was as follows: all patients received 5-fluorouracil (FU) (330 mg/m^2 per day) administrated continuously for 24 h from day 1 to day 5 and day 8 to day 12, and either interferon (IFN)-α-2b or pegylated (PEG) IFN-α-2b used at the treating physician's discretion. PEG IFN-α-2b (1.0 μg/kg) was administrated s.c. on days 1, 8, 15 and 22, and IFN-α-2b (3×10^6 U) was administrated i.m. thrice weekly. Some patients underwent cisplatin administration (20 mg/m^2 per day) into the hepatic artery for 10 min prior to 5-FU. A treatment cycle consisted of 28 days of drug administration, followed by a 14-day rest period. The treatment was repeated until tumor progression or unacceptable toxicity was observed, or until the patient refused the treatment. The treatment protocol was approved by the ethics Committee of Kanazawa University, and informed consent for participation in the study was obtained from each subject and conformed to the guidelines of the 1975 Declaration of Helsinki.

Data collection

We reviewed the medical records of the patients, and collected demographic, clinical and laboratory data, including patient age, sex, ECOG PS, history of viral infection, hepatic reserve (Child–Pugh score), imaging data (vascular invasion and extrahepatic lesion) and tumor marker analyses. We collected laboratory data on complete blood count and CRP. The NLR was calculated

from the differential leukocyte count by dividing the absolute neutrophil count by the absolute lymphocyte count. We used the laboratory data obtained within 7 days prior to day 1 of treatment in this study. We also collected NLR values at 4 weeks after the treatment began to evaluate the impact of the NLR trend on patient outcomes. Cytokine and chemokine profiling was obtained as described below:[19] after venous blood was centrifuged at 1580 *g* for 10 min at 4°C, serum fractions were obtained and stored at −20°C until used. Serum levels of various cytokines and chemokines were measured using the Bio-Plex Protein Array System (Bio-Rad, Richmond, CA, USA) according to the manufacturer's protocol. Briefly, frozen serum samples were thawed at room temperature, diluted 1:4 in sample diluents, and 50 μL aliquots of diluted sample were added in duplicate to the wells of 96-well microtiter plates containing the coated beads for a validated panel of human cytokines and chemokines according to the manufacturer's instructions. The following 20 cytokines and chemokines were targeted: epidermal growth factor (EGF), basic fibroblast growth factor, hepatocyte growth factor, IFN-γ, interleukin (IL)-2, IL-4, tumor necrosis factor-α (TNF-α), IL-6, IL-8, IL-10, IL-5, IFN γ-induced protein (IP)-10, monokine induced by IFN-γ (MIG), platelet-derived growth factor (PDGF)-BB, transforming growth factor (TGF)-β, TGF-α, vascular endothelial growth factor (VEGF), stem cell factor, IL-12 and stromal cell-derived factor 1. Nine standards (range, 0.5–32 000 pg/mL) were used to generate calibration curves for each cytokine. Data acquisition and analysis were performed using Bio-Plex Manager software version 4.1.1 (Bio-Rad).

Evaluation of antitumor effect

The efficacy of HAIC was assessed every 4–6 weeks by dynamic CT or dynamic MRI during the treatment period. The response to chemotherapy was assessed by treating physicians according to the Response Evaluation Criteria in Solid Tumors version 1.1.[20] An objective response rate was defined as the sum of complete response rate and partial response rate.

Statistical analysis

We compared patient backgrounds according to NLR and patient demographics using the χ^2-test for categorical variables when appropriate. Student's *t*-test and Mann–Whitney *U*-test were used for continuous variables. We set the cut-off level of continuous variables as the median value among all patients in this study. We divided the patients into two groups according to NLR before and after treatment, respectively, and compared the response to HAIC and patient outcome between groups. The χ^2-test was also used to evaluate the relation between NLR and the response to HAIC in univariate analysis. Logistic regression analysis was used for multivariate analysis. Progression-free survival (PFS) was calculated from the first day of HAIC until the date of radiological progression, death or the last day of the follow-up period. Overall survival (OS) was calculated from the first day of HAIC until the date of death or the last day of the follow-up period. To compare PFS and OS between groups, the cumulative survival proportions were calculated using the Kaplan–Meier method, and any differences were evaluated using the log–rank test. Only variables that achieved statistical significance in the univariate analysis were subsequently evaluated in the multivariate analysis using Cox's proportional hazards regression model. Linear regression was used to explore the relationship between cytokine or chemokine profiling and NLR. A *P*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS statistical software program package (SPSS, Chicago, IL, USA).

RESULTS

Patients characteristics stratified by NLR

WE RETROSPECTIVELY LISTED 267 consecutive patients who met the above-described criteria and reviewed their medical records. The information regarding the differential leukocyte count could not be obtained in one patient, and then the remaining 266 patients were analyzed. One hundred and thirty-three (50.0%) of 266 patients had NLR higher than 2.87, the median value among all patients before treatment. Patient demographic characteristics are summarized in Table 1. Patients with high NLR had a significantly worse performance status than those with low NLR ($P = 0.020$). With regard to tumor status, vascular invasion and extrahepatic dissemination were observed more often in the patients with high NLR (57.1% and 27.8%, respectively) than in those with low NLR (39.8% and 18.0%, respectively), and des-γ-carboxyprothrombin (DCP) was higher in the group with high NLR (median, 1286 mAU/mL) than in the one with low NLR (median, 214 mAU/mL). Sorafenib was administrated as prior treatment before HAIC in 25 patients (9.4%) and as subsequent therapy after HAIC in 26 patients (9.8%). The proportion of the patients receiving sorafenib before HAIC was similar between the two groups, whereas the proportion of the patients

Table 1 Clinical characteristic of the patients according to NLR

	All ($n = 266$)	High NLR ($n = 133$)	Low NLR ($n = 133$)	P
Age, years				<0.01*
Mean ± SD	66.3 ± 9.1	64.7 ± 9.9	68.0 ± 7.8	
Sex, *n* (%)				0.30**
Male	209 (78.6)	108 (81.2)	101 (75.9)	
ECOG PS, *n* (%)				0.020**
0	220 (82.7)	103 (77.4)	117 (88.0)	
1	41 (15.4)	25 (18.8)	16 (12.0)	
2	5 (1.9)	5 (3.8)	0	
Sorafenib before HAIC				0.83**
Present	25 (9.4)	12 (9.0)	13 (9.8)	
Sorafenib after HAIC				0.013**
Present	26 (9.8)	19 (14.3)	7 (5.3)	
HBs antigen, *n* (%)				0.27**
Positive	70 (26.3)	39 (29.3)	31 (23.3)	
HCV antibody, *n* (%)				<0.01**
Positive	146 (54.9)	57 (42.9)	89 (66.9)	
Child–Pugh score, *n* (%)				0.34**
5–6	134 (50.4)	61 (45.9)	73 (54.9)	
7	55 (20.7)	30 (22.6)	25 (18.8)	
8–9	77 (28.9)	42 (31.6)	35 (26.3)	
Vascular invasion, *n* (%)				<0.01**
Positive	129 (48.5)	76 (57.1)	53 (39.8)	
Extrahepatic lesion, *n* (%)				0.058**
Positive	61 (22.9)	37 (27.8)	24 (18.0)	
CRP, mg/dL				<0.01*
Mean ± SD	1,9 ± 3.0	2.8 ± 3.8	0.9 ± 1.2	
AFP, ng/mL				0.41***
Median, range	241.5, <10–1 637 200	312.5, <10–745 900	119.5, <10–1 637 200	
DCP, mAU/mL				<0.01***
Median, range	567, <10–1 208 000	1 286, <10–1 208 000	214, <10–326 300	

*Student's *t*-test, **χ^2-test, ***Mann–Whitney *U*-test.
AFP, α-fetoprotein; CRP, C-reactive protein; DCP, des-γ-carboxyprothrombin; ECOG PS, Eastern Cooperative Oncology Group performance status; HBs antigen, hepatitis B surface antigen; HCV antibody, hepatitis C virus antibody; NLR, neutrophil to lymphocyte ratio; SD, standard deviation.

receiving sorafenib after HAIC was higher in the group with high NLR (14.3%) than in the one with low NLR (5.3%) ($P = 0.013$).

Treatment

The data collection cut-off was 20 April, 2014. The median follow-up period was 11.4 months (range, 0.3–127.6). At the time of the analysis, 212 patients (79.7%) had died. A total of 715 courses were administrated to 266 patients, with a median number of two (range, 0–13). All but 18 patients including 12 patients (9.0%) in the high NLR group and six (4.5%) in the low NLR group completed at least one course of HAIC.

Of the 266 patients, IFN-α-2b and PEG IFN-α-2b was used in 131 patients (49.2%) and 135 patients (50.8%), respectively. The response to HAIC and the patient outcomes were similar between the different IFN groups. Cisplatin was administrated in 186 patients (69.9%). Although response to HAIC had a tendency to be better in patients in the cisplatin group than those of the patients without cisplatin, there was no significant differences of the treatment efficacies.

Response to HAIC and PFS stratified by pretreatment NLR

Of the 266 patients, 15 patients could not receive radiological assessment because of worsened general condition, hepatic failure or loss to follow up, and the remaining 251 were assessable for response to treatment. The tumor responses to HAIC are shown in

Table 2 Tumor responses according to NLR

Response* to HAIC	All ($n = 266$)	High NLR ($n = 133$)	Low NLR ($n = 133$)
CR	16 (6.0)	3 (2.3)	13 (9.8)
PR	62 (23.3)	25 (18.8)	37 (27.8)
SD	83 (31.2)	40 (30.1)	43 (32.3)
PD	90 (33.8)	55 (41.4)	35 (26.3)
NE	15 (5.6)	10 (7.5)	5 (3.8)
Objective response rate	29.3%	21.1%	37.6%
	$P < 0.01$**		

*RECIST version 1.1, **χ^2-test.
Data are presented as *n* (%).
CR, complete response; HAIC, hepatic arterial infusion chemotherapy; NE, not evaluated; NLR, neutrophil to lymphocyte ratio; PD, progressive disease; PR, partial response; SD, stable disease.

Table 2. The objective response rate was 37.6% in patients with low NLR, which was significantly better than that of the patients with high NLR (21.1%; $P < 0.01$). Multivariate logistic regression analysis revealed that low NLR (hazard ratio [HR], 1.918; $P = 0.024$) as well as vascular invasion (HR, 1.874; $P = 0.029$) and extrahepatic lesion (HR, 2.723; $P = 0.012$) remained independently associated with the response to HAIC (Table 3).

The median PFS of all patients was 4.5 months. The PFS of patients with high NLR was shorter than that of the patients with low NLR, and the median PFS of the patients with high NLR was 3.2 months, which was significantly worse than that of the patients with low NLR of 5.6 months (Fig. 1a). The following nine of the

Table 3 Pretreatment factors affecting objective response

		n	ORR (%)	Univariate *P**	Hazard ratio (95% CI)	Multivariate *P***
NLR	<2.87	133	37.6	<0.01	1.918 (1.092–3.369)	0.024
	≥2.87	133	21.1			
Age, years	≥67	136	31.6	0.40		
	<67	130	26.9			
Sex	Male	209	29.7	0.81		
	Female	57	28.1			
ECOG PS	0	220	32.3	0.051		
	1	41	17.1			
	2	5	0			
Prior treatment of sorafenib	Absence	241	29.5	0.88		
	Presence	25	28.0			
HBs antigen	Positive	70	32.9	0.45		
	Negative	196	28.1			
HCV antibody	Positive	146	31.5	0.39		
	Negative	120	26.7			
Child–Pugh score	5–6	134	35.8	0.054		
	7	55	25.5			
	8–9	77	20.8			
Vascular invasion	Absence	137	36.5	<0.01	1.874 (1.067–3.292)	0.029
	Presence	129	21.7			
Extrahepatic lesion	Absence	205	33.7	<0.01	2.723 (1.250–5.932)	0.012
	Presence	61	14.8			
CRP, mg/dL	<0.8	127	33.9	0.11		
	≥0.8	136	25.0			
AFP, ng/mL	<235.5	133	31.6	0.42		
	≥235.5	133	27.1			
DCP, mAU/mL	<567	133	33.8	0.11		
	≥567	133	24.8			

*χ^2-Test, **logistic regression analysis.
AFP, α-fetoprotein; CI, confidence interval; CRP, C-reactive protein; DCP, des-γ-carboxyprothrombin; ECOG PS, Eastern Cooperative Oncology Group performance status; HBs antigen, hepatitis B surface antigen; HCV antibody, hepatitis C virus antibody; NLR, neutrophil to lymphocyte ratio; ORR, objective response rate.

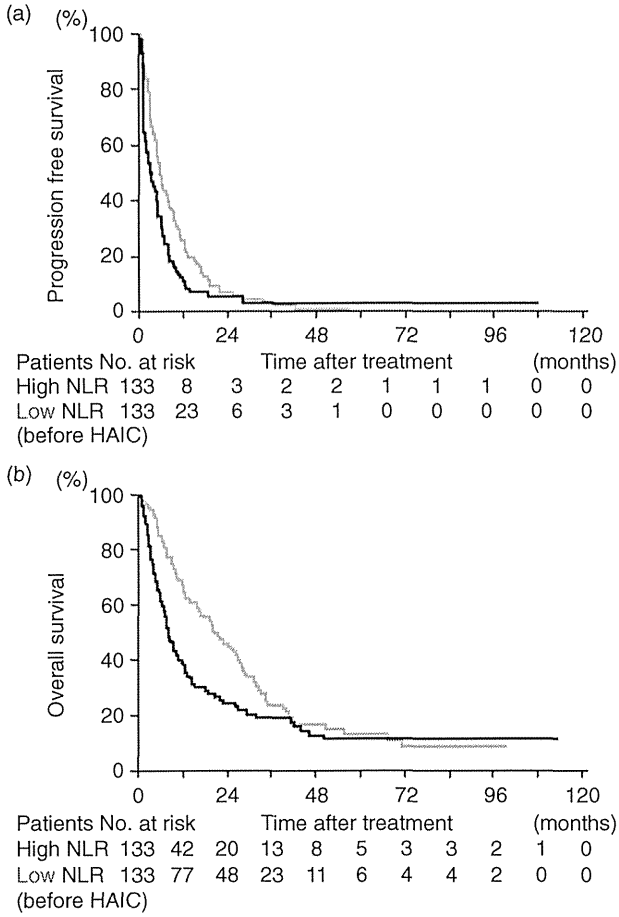

Figure 1 Kaplan–Meier plot of progression-free survival (PFS) and overall survival (OS) since commencement of HAIC according to neutrophil to lymphocyte ratio (NLR). (a) Median PFS of the patients with high NLR was 3.2 months, which was significantly worse than that of the patients with low NLR, 5.6 months ($P < 0.01$). (b) Median OS of the patients with high NLR was 8.0 months, which was significantly worse than that of the patients with low NLR, 20.7 months ($P < 0.01$). —, High NLR; ·····, Low NLR.

12 pretreatment variables were significantly associated with the PFS times in univariate analyses: ECOG PS ($P < 0.01$), hepatitis B surface antigen (HBsAg; $P < 0.01$), hepatitis C virus antibody ($P = 0.044$), vascular invasion ($P < 0.01$), extrahepatic lesion ($P < 0.01$), CRP ($P < 0.01$), α-fetoprotein (AFP) ($P < 0.01$) and DCP ($P < 0.01$) as well as NLR. Pretreatment high NLR was an independent unfavorable factor for PFS (HR, 1.363; $P = 0.044$) as well as ECOG PS 1 and 2 (HR compared with ECOG PS, 1.585; $P = 0.019$ and 3.301; $P = 0.025$, respectively), HBsAg positive (HR, 1.687; $P < 0.01$), extrahepatic lesion (HR, 1.500; $P = 0.025$) and AFP of 235.5 ng/mL or more (HR, 1.580; $P < 0.01$) in Cox's proportional hazards regression model (Table 4).

Patient outcome stratified by pretreatment NLR

The median OS of all patients was 12.6 months. The OS in the patients with high NLR was shorter than that of the patients with low NLR ($P < 0.01$), and the median OS in the patients with high NLR was 8.0 months, which was significantly worse than that of the patients with low NLR (20.7 months) (Fig. 1b). The following eight of the 12 pretreatment variables were significantly associated with the OS in univariate analyses: ECOG PS ($P < 0.01$), Child–Pugh score ($P < 0.01$), vascular invasion ($P < 0.01$), extrahepatic lesion ($P < 0.01$), CRP ($P < 0.01$), AFP ($P < 0.01$) and DCP ($P < 0.01$) as well as NLR. Pretreatment high NLR was an independent unfavorable factor for OS (HR, 1.492; $P < 0.01$) as well as ECOG PS 1 and 2 (HR compared with ECOG PS 0, 1.597; $P = 0.034$ and 3.825; $P = 0.013$, respectively), Child–Pugh score 8 or 9 (HR compared with Child–Pugh score 5 or 6, 1.454; $P = 0.036$), extrahepatic lesion (HR, 1.677; $P < 0.01$), CRP of 0.8 or more (HR, 1.406; $P = 0.031$) and AFP of 235.5 or more (HR, 1.702; $P < 0.01$) in Cox's proportional hazards regression model (Table 5).

Patient outcome according to trend of NLR

We obtained the NLR value at 4 weeks after the start of HAIC in 243 patients. Of the patients with high NLR before HAIC ($n = 120$), NLR was low at 4 weeks after the start of HAIC (High-Low) in 69 patients (57.5%). The median PFS in the patients with High-Low was 4.9 months, which was significantly better than that of the patients with high NLR at 4 weeks after the start of HAIC (High-High), 2.0 months ($P = 0.030$). The median OS in the patients with High-Low was 11.5 months, which was significantly better than that of the patients with High-High, 6.1 months ($P < 0.01$) (Fig. 2a). In contrast, of the patients with low NLR before HAIC ($n = 123$), NLR was high at 4 weeks after the start of HAIC (Low-High) in 11 (8.9%) patients. The median PFS in the patients with Low-High was 2.0 months, which was significantly worse than that of the patients with low NLR at 4 weeks after the start of HAIC (Low-Low), 6.0 months ($P < 0.01$). The median OS in the patients with Low-High was 5.5 months, which was significantly worse than that of the patients with Low-Low, 22.6 months ($P < 0.01$) (Fig. 2b).

Table 4 Pretreatment factors affecting progression-free survival

		n	mPFS (months)	Univariate *P**	Hazard ratio (95% CI)	Multivariate *P***
NLR	≥2.87	133	3.2	<0.01	1.363 (1.008–1.843)	0.044
	<2.87	133	5.6			
Age, years	<67	130	4.0	0.46		
	≥67	136	5.2			
Sex	Male	209	4.5	0.31		
	Female	57	5.1			
ECOG PS	2	5	0.9	<0.01	3.301 (1.165–9.355)	0.025
	1	41	2.7		1.585 (1.079–2.330)	0.019
	0	220	4.9			
Prior treatment of sorafenib	Absence	241	4.5	0.95		
	Presence	25	4.8			
HBs antigen	Positive	70	2.5	<0.01	1.687 (1.163–2.447)	<0.01
	Negative	196	5.5			
HCV antibody	Negative	120	3.1	0.044	0.841 (0.596–1.188)	0.33
	Positive	146	5.5			
Child–Pugh score	8–9	77	3.2	0.099		
	7	55	4.5			
	5–6	134	5.1			
Vascular invasion	Presence	129	2.7	<0.01	1.191 (0.876–1.619)	0.27
	Absence	137	6.2			
Extrahepatic lesion	Presence	61	2.8	<0.01	1.500 (1.053–2.138)	0.025
	Absence	205	5.5			
CRP, mg/dL	≥0.8	136	2.8	<0.01	1.293 (0.952–1.758)	0.10
	<0.8	127	6.2			
AFP, ng/mL	≥235.5	133	2.8	<0.01	1.580 (1.162–2.148)	<0.01
	<235.5	133	6.2			
DCP, mAU/mL	≥567	133	3.2	<0.01	1.203 (0.873–1.659)	0.26
	<567	133	5.6			

*Log-rank test, **Cox's proportional hazards regression model.
AFP, α-fetoprotein; CI, confidence interval; CRP, C-reactive protein; DCP, des-γ-carboxyprothrombin; ECOG PS, Eastern Cooperative Oncology Group performance status; HBs antigen, hepatitis B surface antigen; HCV antibody, hepatitis C virus antibody; mPFS, median progression-free survival time; NLR, neutrophil to lymphocyte ratio.

Correlation between cytokine or chemokine profiling and NLR

Data of cytokine and chemokine profiling were obtained in 86 patients. We investigated the association between the value of cytokine or chemokine and NLR to analyze the mechanisms of NLR to cancer biology. Results are shown in Table 6. Serum PDGF-BB concentration had a significant positive correlation with NLR ($r = 0.227$; $P = 0.035$) (Fig. S1). No other cytokine or chemokine was correlated with NLR.

DISCUSSION

THE FIRST AIM of this study was to investigate the correlation between NLR and patient characteristics in advanced HCC. Some reports have suggested that NLR is correlated with tumor biology in unselected cohorts of patients with HCC.[21] Our analysis also demonstrated the corresponding results in patients with HCC at an advanced stage. Moreover, it was newly clarified that NLR had a strong relation with ECOG PS, which was an important factor reflecting a variety of complications of liver cirrhosis or tumor-related symptoms.[22]

The most important insight of our study was that NLR was correlated with the treatment efficacies presented as response to HAIC or PFS as well as patient outcome given that this is the largest cohort of patients with advanced HCC treated with HAIC, to the best of our knowledge. Our results should be interpreted with caution because of the bias introduced by the differences of patient characteristics observed between the

Table 5 Pretreatment factors affecting overall survival

		n	mOS (months)	Univariate *P**	Hazard ratio (95% CI)	Multivariate *P***
NLR	≥2.87	133	8.0	<0.01	1.492 (1.106–2.012)	<0.01
	<2.87	133	20.7			
Age, years	<67	130	9.9	0.18		
	≥67	136	17.7			
Sex	Female	57	10.7	0.091		
	Male	209	13.6			
ECOG PS	2	5	2.4	<0.01	3.825 (1.329–11.009)	0.013
	1	41	7.3		1.597 (1.035–2.463)	0.034
	0	220	14.5			
Prior treatment of sorafenib	Presence	25	11.6	0.77		
	Absence	241	13.1			
HBs antigen	Positive	70	8.4	0.095		
	Negative	196	15.4			
HCV antibody	Negative	120	10.7	0.096		
	Positive	146	16.6			
Child-Pugh score	8–9	77	6.9	<0.01	1.454 (1.024–2.064)	0.036
	7	55	13.7		0.942 (0.621–1.429)	0.78
	5–6	134	16.6			
Vascular invasion	Presence	129	8.2	<0.01	1.138 (0.819–1.582)	0.44
	Absence	137	19.6			
Extrahepatic lesion	Presence	61	6.5	<0.01	1.677 (1.144–2.458)	<0.01
	Absence	205	16.6			
CRP, mg/dL	≥0.8	136	8.7	<0.01	1.406 (1.031–1.917)	0.031
	<0.8	127	22.6			
AFP, ng/mL	≥235.5	133	8.7	<0.01	1.702 (1.228–2.359)	<0.01
	<235.5	133	21.8			
DCP, mAU/mL	≥567	133	9.0	<0.01	1.123 (0.808–1.568)	0.49
	<567	133	20.7			

*Log-rank test, **Cox's proportional hazards regression model.
AFP, α-fetoprotein; CI, confidence interval; CRP, C-reactive protein; DCP, des-γ-carboxyprothrombin; ECOG PS, Eastern Cooperative Oncology Group performance status; HBs antigen, hepatitis B surface antigen; HCV antibody, hepatitis C virus antibody; mOS, median overall survival time; NLR, neutrophil to lymphocyte ratio.

high NLR group and low NLR group. However, our results suggested that NLR was a predictor of response to HAIC in multivariate analysis independent of ECOG PS, hepatic reserve and tumor-related factors in this study. CRP was suggested as a prognostic marker for patients with HCC treated with sorafenib;[23] however, it remains unclear whether such factors can predict antitumor effects of sorafenib or the prognosis of patients with advanced HCC. NLR may be a stronger predictor than CRP of both of antitumor effects and prognosis of patients with advanced HCC treated with HAIC. The differential leukocyte count is an inexpensive and routinely measured marker in daily clinical practice and, therefore, NLR is a simple and easily available marker for the selection of suitable patients to undergo HAIC.

Another interesting point of the present study was that the cumulative survival curve was stratified according to trend of NLR before and after HAIC. The antitumor effect was evaluated generally by radiological findings and the trends of tumor markers, such as AFP or DCP in HCC.[24] However, these modalities have disadvantages such as complications, cost of measurements and lack of universality because the evaluation was often difficult to interpret.[25] Further, tumor markers were not elevated in one-third of the patients with HCC.[17] Our findings suggested that NLR, a simple and economical marker derived from routinely available blood tests, was helpful in evaluating the efficacy of HAIC or predicting the outcomes of the patients with advanced HCC by following its trend.

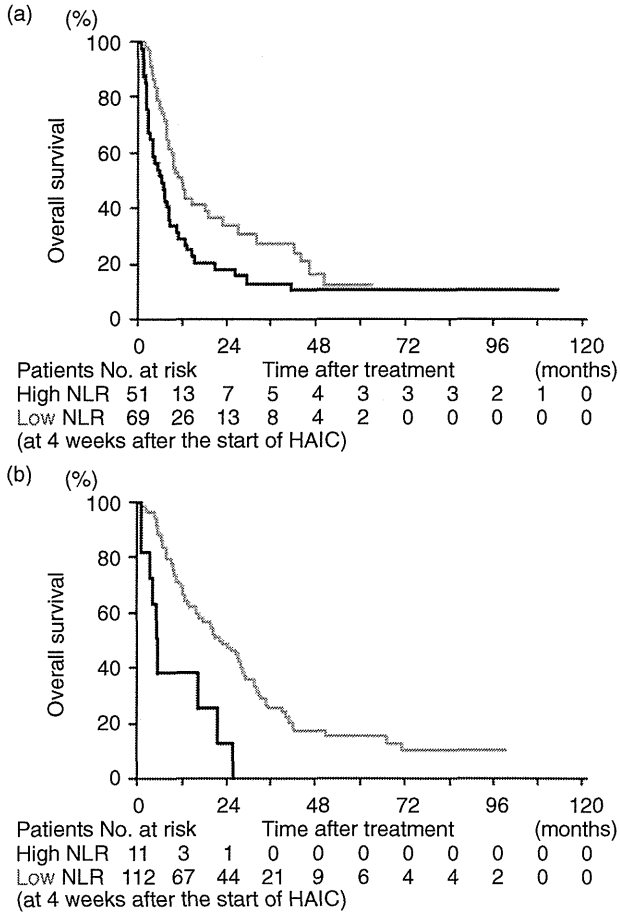

Figure 2 Kaplan–Meier plot of overall survival (OS) since commencement of hepatic arterial infusion chemotherapy (HAIC) according to neutrophil to lymphocyte ratio (NLR) at 4 weeks after the start of treatment. (a) Among the patients with high NLR before HAIC, median OS of the patients whose NLR was reduced (High-Low) was 11.5 months, which was significantly better than that of the patients with remaining high NLR (High-High), 6.1 months ($P < 0.01$). (b) Among the patients with low NLR before HAIC, median OS of the patients whose NLR was elevated (Low-High) was 5.5 months, which was significantly worse than that of the patients with remaining low NLR (Low-Low), 22.6 months ($P < 0.01$).

Finally, our findings indicated that PDGF-BB was a candidate of mediators for NLR, reflecting tumor biology and response to HAIC. It was reported that activated neutrophils stimulate the growth and progression of the cancer cells by releasing growth factors such as PDGF-BB.[26] It has been shown that PDGF-BB also promotes angiogenesis and subsequent vascular invasion[27] and may reduce the sensitivity to cytotoxic agents in HCC.[28] Some reports stated that the serum level of PDGF-BB correlated with the efficacy of treatments for HCC,[27,29] and should be paid more attention when considering treatment of patients with HCC.

The present study has several limitations. For instance, the study was retrospective in nature and it was conducted at a single center. Therefore, further study is needed to validate our findings.

In conclusion, high NLR was strongly correlated with poor general condition and advanced tumor progression in patients with advanced HCC. NLR can act as a predictive and prognostic factor for patients with advanced HCC treated with HAIC. The trends of NLR after treatment of HAIC strongly reflected the patient outcomes in this study. Our findings can be useful in determining treatment strategies or in designing future clinical chemotherapy trials of advanced HCC.

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Table 6 Association between cytokine or chemokine and NLR

	r	*P**
EGF	0.001	0.99
FGF	0.141	0.20
HGF	0.011	0.92
IFN-γ	0.132	0.23
IL-2	0.103	0.35
IL-4	0.161	0.14
TNF-α	0.124	0.26
IL-6	0.159	0.15
IL-8	−0.080	0.47
IL-10	0.121	0.27
IL-5	−0.035	0.75
IP10	−0.089	0.42
MIG	−0.112	0.31
PDGF-BB	0.227	0.035
TGF-β	0.000	1.00
TGF-α	−0.041	0.71
VEGF	−0.102	0.35
SCF	−0.088	0.42
IL-12	0.040	0.71
SDF-1	−0.077	0.48

*Linear regression.
EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; IP, interferon-γ-induced protein, MIG, monokine induced by interferon-γ; PDGF, platelet-derived growth factor; SCF, stem cell factor; SDF, stromal cell-derived factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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